EFFECTS OF FERTILIZER ON ROOT NODULATION, CHLOROPHYLL CONTENT AND NITRATE REDUCTASE ACTIVITY IN LONG BEAN AND MUNG BEAN

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ABSTRACT

A greenhouse experiment was conducted to investigate the effects of fertilizer on photosynthetic pigments (Chl. *a* and Chl. *b*) and root nodulation pattern in long bean (*Vigna unguiculata sesquipedalis*) and mung bean (*Vigna radiata* L.) under humid tropical conditions in Malaysia. The total chlorophyll, chlorophyll *a*, chlorophyll *b*, chlorophyll *a/b* ratio, photosynthetic rate, seeds yield, total dry matter, number of nodules and pods, nodule dry matter per plant and nitrate reductase activity of nodules in each legume were determined throughout the growing season. The highest total chlorophyll content in leaf (2.99 mg/g and 41.35 SPAD value at flower initiation stage) and photosynthetic rate (1.26 µmol m^{-2} s^{-1} at flower initiation stage) were recorded in long bean grown with fertilizer. Long bean grown with fertilizer showed the highest chlorophyll *a* content (2.25 mg/g) at flower initiation stage whilst mung bean grown without fertilizer possessed the highest content of chlorophyll *b* (0.80 mg/g**)** at flower initiation stage and chlorophyll *a/b* ratio (0.62) at pod formation stage. Long bean grown without fertilizer recorded the highest pod dry matter (1.17 g/plant at maturity stage) and total dry weight (32.93 g/plant at maturity stage). The highest number of nodules per plant (17.33) and the maximum nodule dry weight (0.07 g/plant) were noticed in long bean grown without fertilizer at the onset of flowering. Number of pods and pod dry weight of the crops grown with and without fertilizer were more or less the same. The nitrate reductase activities of nodules in long bean grown without fertilizer possessed the highest activity (17.65 µmols NO_2^- h^{-1} $gfwt^{-1}$) at flower initiation stage whereas mung bean grown with fertilizer possessed the lowest (6.68 µmols NO_2^- h^{-1} $gfwt^{-1}$). This investigation verified that application of NPK fertilizer can reduce the nitrogen fixation efficiency by poor production of nodules and nitrate

reductase activity that is required to maintain soil fertility. The outcome disclosed that long bean possesses abundant photosynthetic pigments and has the remarkable capability in nodules production compared to mung bean.

ABSTRAK

Satu eksperimen rumah hijau telah dijalankan untuk menyiasat kesan baja ke atas pigmen fotosintesis (Klorofil *a* dan Klorofil *b*) dan corak nodulasi pokok kacang panjang (*Vigna unguiculata sesquipedalis*) serta pokok kacang hijau (*Vigna radiata* L.) di bawah cuaca tropika lembap di Malaysia. Jumlah kandungan klorofil, klorofil *a*, klorofil *b*, nisbah klorofil *a/b*, kadar fotosintesis, hasil bijirin, jumlah jisim kering, bilangan nodul dan buah, jisim kering nodul setiap pokok dan aktiviti nitrat reduktase di dalam nodul setiap pokok kekacang telah ditentukan sepanjang musim tumbesaran. Jumlah kandungan klorofil yang tertinggi dalam daun (2.99 mg/g dan 41.35 nilai SPAD pada peringkat permulaan bunga) dan kadar fotosintesis tertinggi (1.26 µmol m^{-2} s^{-1} pada peringkat permulaan bunga) telah direkodkan oleh pokok kacang panjang yang tumbuh dengan baja. Pokok kacang panjang yang tumbuh dengan baja telah menunjukkan kandungan klorofil *a* (2.25 mg/g) pada peringkat permulaan bunga manakala pokok kacang hijau yang tumbuh tanpa baja memiliki kandungan klorofil *b* yang paling tinggi (0.80 mg/g pada peringkat permulaan bunga) serta kandungan nisbah klorofil *a/b* (0.62 pada peringkat pembentukan buah). Pokok kacang panjang yang tumbuh tanpa baja telah merekodkan jisim kering buah tertinggi (1.17 g/pokok pada peringkat kematangan) dan jumlah jisim kering yang tertinggi (32.93 g/pokok pada peringkat kematangan). Bilangan tertinggi nodul (17.33/pokok) dan jisim kering nodul maksimum (0.07 g/pokok) dijumpai dalam pokok kacang panjang tumbuh tanpa baja pada peringkat permulaan bunga. Bilangan buah dan jisim kering buah diantara tanaman tumbuh dengan baja atau tanpa baja tidak ketara. Aktiviti – aktiviti nitrat reduktase dalam nodul pokok kacang panjang tumbuh tanpa baja memiliki aktiviti yang tertinggi pada peringkat permulaan bunga (17.65 µmols NO_2^- jam^{-1} g berat

basah^{-1}) manakala pokok kacang hijau tumbuh dengan baja memiliki aktiviti yang terendah (6.68 µmols NO_2^- jam^{-1} g berat basah^{-1}). Kajian ini telah mengesahkan bahawa penggunaan baja NPK mungkin mengurangkan proses penetapan nitrogen dengan mengurangkan pengeluaran nodul dan aktiviti nitrat reduktase yang diperlukan dalam mengekalkan kesuburan tanah. Hasil mendedahkan bahawa pokok kacang panjang memiliki pigmen fotosintesis yang banyak dan mempunyai keupayaan yang menakjubkan dalam pengeluaran nodul berbanding pokok kacang hijau.

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^0C	degree celsius
&	and
e^-	electron
%	percent
<	less than
0F	zero fertilizer
a.m.	ante meridian
ATP	adenosine triphosphate
Ala	alanine
Asn	asparagine
ADI	acceptable daily intake
Abs	absorbance
ARA	acetylene reduction assay
BNF	biological nitrogen fixation
Ca	calcium
CGR	crop growth rate
Chl	chlorophyll
CO_2	carbon dioxide
CHO	carboxy group
CH_3	methyl group
CHN	carbon, hydrogen, nitrogen
Cl	chlorine

CRD	completely randomized design
DAE	days after emergence
dH_2O	distilled water
ETC	electron transport chain
FAD	flavine adenosine dinucleotide
Fe^{2+}	Ferum ion
Glu	glutamate
g	gram
$gfwt^{-1}$	gram per fresh weight
g/plant	gram per plant
HIV	human immunodeficiency virus
H_2O	water
HNO_3	nitric acid
HCl	hydrochloric acid
h^{-1}	per hour
ICP – OES	Inductively Coupled Plasma – Optical Emission Spectrometry
K	potassium
kDa	kilo dalton
KNO_3	potassium nitrate
LA	leaf area
LAI	leaf area index
Lb	leghaemoglobin
Lci – SD	Leaf Chamber/Soil Respiration Analysis System
µ	micro

Mg	magnesium
Mg^{2+}	magnesium ion
Mo	molybdenum
MoCo	molybdenum cofactor
mg	miligram
mg/g	milligram per gram
mg/L	milligram per litre
mL	mililitre
MARDI	Malaysian Agricultural Research and Development Institute
M	molar
mRNA	messenger ribonucleic acid
N	nitrogen
N_2	dinitrogen
NAR	net assimilation rate
NH_3	ammonia
NH_4	ammonium
NH_4^+	ammonium ion
NS	non-significant
NO_3	nitrate
NO_3^-	nitrate ion
NO_2	nitrite
NO_2^-	nitrite ion
NR	nitrate reductase
NiR	nitrite reductase

NRA	nitrate reductase activity
NEED	naphthylethylene diamine dihydrochloric acid
nm	nanometre
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate
NPK	nitrogen, phosphorus, potassium
Nod^-	non-nodulating
O_2	oxygen molecule
P	phosphorus
ppm	parts per million
R	side chain in molecule structure
RUBISCO	ribulose-1,5-bisphosphate carboxylase oxygenase
RGR	relative crop growth
Ser	serine
SPAD	Soil-Plant Analysis Development
SA	sulfanilamide
S	sulphur
SO_4^{2-}	sulphate ion
SMYRK	symbiosis-receptor-kinase-gene
t	time

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CHAPTER 1

INTRODUCTION

Legume crops are the second largest group of food and feed crops grown globally and cultivated on 12 – 15% of arable land. It is responsible for more than 25% of the world's primary crop production with 247 million tons of grain legumes produced annually (Ferguson *et al.*, 2010). The tropical leguminous plants are distinguished from other plant families by their ability to form N-fixing root nodules in close cooperation with bacteria from the genera *Rhizobium* or *Bradyrhizobium*. Legumes belong to Rhizobiaceae family and are able to form symbiotic mutualistic association with *Rhizobium* (Samac and Graham, 2007; Varges *et al.,* 2000) which play a significant role in biological nitrogen fixation (BNF) (Silverstein *et al.*, 2006). Legume plants have tremendous ability in fixing nitrogen (N_2) and producing residues which are rich in nitrogen (N) that can be absorbed by plants from the soil (Giller, 2001; Hirsch, 2009). Nitrogen utilization from the soil is required for plant productivity. Approximately 80 – 110 kg ha^{-1} of the total plant N is supplied through the N_2 fixation process. In BNF, the carbon for N-fixing bacteria is provided by plants and the oxygen-sensitive nitrogenase enzyme is required for the conversion of dinitrogen (N_2) to ammonium (NH_4). Nitrate reductase is also one of the enzymes which are vital in the overall N metabolism in plants that is responsible in the reduction of nitrate (NO_3) to nitrite (NO_2). Nitrate is acknowledged as the primary source of N in the soil.

Nitrate reductase (NR) is the enzyme which reduces NO_3 to NO_2. This cytosolic enzyme is present in leaves and roots of higher plants (Chung *et al.*, 2004). In legume plant, nodules are developed when rhizobia invade the roots. The bacteria then

differentiate into bacteroids within the nodules and nitrogenase enzyme complex catalyzes the conversion of atmospheric N to NH_3 (Hirsch, 2009; Ferguson *et al.*, 2010), which is essential for crop growth and plant yield. In this interaction, rhizobia provide atmospheric N to the protein-rich seeds and legumes which are important in human and animal diets (Samac and Graham, 2007; Ferguson *et al*, 2010). Nitrogen is responsible in increasing dry matter of crops (Reynolds, 2005) and crop growth. Deficiency of N is the main factor that limits bean production in most countries which leads to stunted growth, low quality of leaf and yellowish leaves, decrease leaf area and photosynthesis intensity (Bojović and Marković, 2009). Many countries encountered this matter by using N fertilizers that contribute to high expenses and adversely affect the nature. In plant growth and development, N is the key limiting factor because it is involved in the production of essential constituent of proteins and enzymes. It is also involved in metabolic processes such as in the synthesis and transfer of energy, vitamins, nucleic acids and in other organic molecules such as chlorophyll which is required for photosynthesis (Wagner, 2012).

Photosynthesis is a process where chlorophylls in the chloroplasts harvest light energy from the sun and convert it to chemical energy which are then stored in sugar and other organic molecules. The rate of plant growth depends on the rate of photosynthesis. There are about half a million chloroplasts per square millimeter of leaf surface (Campbell and Reece, 2005). Nitrogen is important for the synthesis of amino acids, proteins, NADH, ATP, carbohydrates and lipids (Ophardt, 2003). Lack of N will lead to the suppression of chlorophyll formation since this nutrient is an essential element in the chlorophyll molecule. Chlorophyll is the light energy receptor

that contains N. This crucial pigment also plays a role as an index of plant growth and production of organic matter (Lahai *et al.*, 2003). Most plants possess chlorophyll *a* and chlorophyll *b*. Generally, chlorophyll *a* is the main photosynthetic pigment (Campbell and Reece, 2005) and chlorophyll *b* is better known as accessory pigment. Chlorophyll content of leaf tissue is a good indicator of photosynthetic activity (Chowdhury and Kohri, 2003) and significant to manage chemical and fertilizer application (Haboudane *et al.*, 2002; Wu *et al.*, 2008). As a result, chlorophyll content is an important index to physiological activities in plants.

In agricultural systems, N is known as the imperative factor in plant growth and is a major constituent of chlorophyll, amino acids, proteins and photosynthetic activity. Potassium (K) governs fruit quality improvement, plant physiology, increases disease resistance, prevents lodging and improves the capability of plants in tolerating moisture stress. Phosphorus (P) plays a role in development of fruit, root and flower. Uptake of these nutrients may affect qualitatively and quantitatively the growth and fruit yield (Rathore *et al.*, 2008). Ideal conditions such as N availability, soil condition and also field cropping history are required for initiation of BNF which leads to optimum nodulation. In modern agriculture, huge pressure has mounted on the environment due to the usage of chemical fertilizers and pesticides to increase the crops productivity. These activities give rise to environmental problems (Lawlor *et al.*, 2001) especially in relation to NO_3 loss in the environment. When N input exceeds the demand, the sources of N that build up in the soil are mostly NO_3 (Virk, 2014). In general, only a little amount of the NO_3 will be taken up by the plant roots. Accumulation of NO_3 in plants arises from the uptake of nitrate ion (NO_3^-) in excess of

its reduction and subsequent assimilation in the metabolism. The excess NO_3 in plants will accumulate mostly in vacuoles of both shoots and roots. Both environmental friendly BNF technology and green manure are the best method to promote nodule production and improve NO_3 uptake by plants. In the present study, tropical legumes including long bean (*Vigna unguiculata sesquipedalis)* and mung bean (*Vigna radiata* L.*)* were evaluated in order to observe the effect of NPK fertilizer on the photosynthetic pigments, photosynthetic rate and nodulation activities. Such information is required to obtain a more reliable theory and to improve the current understanding of the role of symbiotic rhizobia.

Objectives

The objectives of this study are:

i) To study the nodulation pattern at different growth stages of long bean and mung bean grown with and without fertilizer.

ii) To determine chlorophyll content, photosynthetic rate and dry matter accumulation of long bean and mung bean grown with and without fertilizer.

iii) To determine the nitrate reductase activities in nodules of long bean and mung bean grown with and without fertilizer at different growth stages.

CHAPTER 2

LITERATURE REVIEW

2.1 Leguminosae family

Leguminosae (also known as Fabaceae) family is one of the three largest families of angiosperms with approximately 730 genera and about 19,400 species (Wojciechowski *et al.,* 2004) of shrubs, herbs, climbers and trees. Leguminosae forms a symbiotic relationship with specific soil bacteria, called rhizobia (Genera *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*). These organisms have been known to accumulate N in the soil. The tropical leguminous plants are distinguished from other plant families by their ability to form N-fixing root nodules in close cooperation with bacteria from the genera *Rhizobium* or *Bradyrhizobium*. They are grown globally and cultivated on 12 – 15% of available arable land. They are responsible for more than 25% of the world's primary crop production with 247 million tons of grain legumes produced annually (Ferguson *et al.*, 2010) and present a wealth of resources for utilization (Doyle and Luckow, 2003). Many species of this family are harvested for fuel, timber, oil, medicine, chemicals and as crops (Wojciechowski *et al.,* 2004).

2.1.1 Legumes

Legumes, plants of Leguminosae family, are rich in natural products such as flavonoids, isoflavones and isoflavanones, proteins, mineral nutrients and oils that contribute to human nutrition and health (Doyle and Luckow, 2003). Legume represents the third largest group of angiosperms and the second largest group of food

and feed crops grown globally including pea, clover, chickpea, lentil, alfalfa, mung bean and soybean (Ferguson *et al.,* 2010) which are important for restoring soil fecundity (Hirsch, 2009). Legumes serve as the primary N source and are important in agriculture as the source of food for domestic animals as well as humans after the grasses (Poaceae) (Wojciechowski *et al.*, 2004).

Legumes have the remarkable ability for fixing N_2 and producing residues which are rich in N that can be absorbed by plants from soil (Giller, 2001; Hirsch, 2009). Utilization of N from the soil and seeds are required for plant productivity. About 25 – 30% of the total plant N (80 – 110 kg N per hectare per season) is supplied through the N_2 fixation process (Harper, 1971). Through symbiosis with soil microbes, legumes act as soil-improving components of agroforestry systems and enhance agricultural ecosystems. They are able to gratify large demand of N through assimilation and absorption of inorganic N from the soil for crop plants (International Atomic Energy Agency, 2008). The leguminous plants along with *Rhizobium* in nodules are responsible for converting atmospheric N to NH_3 in N accumulation process where nitrogenase enzyme is involved. Many bacteria possess hydrogenases to oxidize hydrogen in a reaction coupled to N_2 fixation. In the absence of N, nitrogenase produces hydrogen when ATP is supplied (Hirsch, 2009).

2.2 Nitrogen fixation by legumes

Legumes are able to form symbiotic mutualistic association with certain bacteria (Varges *et al.,* 2000; Samac and Graham, 2007) from the Rhizobiaceae family (Silverstein *et al.,* 2006) in BNF. In the presence of BNF, the legume crops' needs

towards inorganic N fertilizers input may be reduced by providing sufficient quantities of N during the growth season (van Kessel and Hartley, 2000). The symbiotic relationship between legumes and rhizobia has an enormous impact on agricultural ecosystems (Samac and Graham, 2007), since legumes fix N through root nodules (Doyle and Luckow, 2003). This phenomenon creates inducement among researchers to investigate the unique nodulation in the legume plants.

Both environmental friendly BNF technology and green farm yard manure are the best methods to promote nodule production and improve NO_3 uptake by plants. This can also lead to alleviation of the accumulation of NO_3 in leafy vegetables (Zhou *et al.*, 2000). The symbiosis initiates once the rhizobia invade the roots of leguminous plants and fix N_2 and provide NH_3 to the legume host plant. This is because the crop cannot take up atmospheric N as essential N element which is required in plant development and growth (Franche *et al.*, 2009; Ferguson *et al.*, 2010).

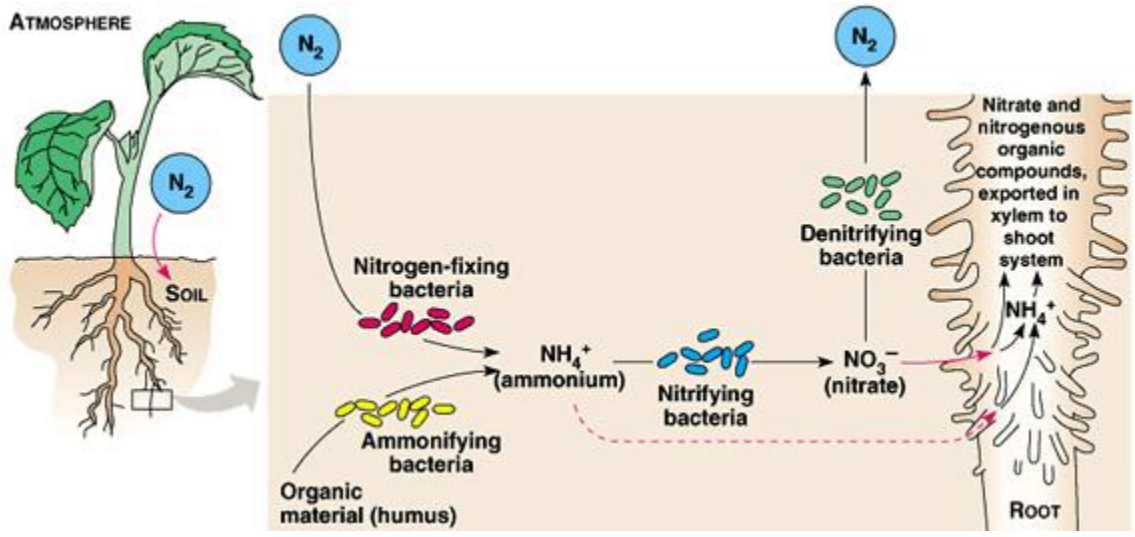

Figure 2.1: Nitrogen fixation by leguminous plant (Campbell and Reece, 2005).

2.3 Other uses of legumes

The use of leguminous crops as green manure is known as a common farming practice in order to rotate species such as alfalfa, mung bean or clover (Ferguson *et al.*, 2010). Thus, organic N content of the soil are enhanced excellently. Legumes are high source of protein for animals and human where complete protein nutrition can be achieved when cereals and legumes are consumed together (Gulmezoglu and Kayan, 2011).

Grain legumes are the second source of animal and human food after cereals and two to three folds richer in protein content. Chickpeas and lentils are both important in Southern Asia, Middle East and North Africa while kidney beans and other legumes are essential legumes in Latin America. Soybeans and peanuts are examples of several legumes rich in oil. The peanut butter, soymilk and tofu are the common products that can be manufactured from these legumes. There is an increase in demand of animal products such as eggs, meat, milk and butter due to enhancement of human nutrition quality in all countries. This demand can be met by using high protein content as animal feeds where soybeans are used the most. Beans contain fibers which are required for digestive system to function smoothly than most fruits and vegetables. Legumes contain antioxidant, folic acid, vitamin C and have anti-carcinogen properties (Khalighi-Sigaroodi *et al.*, 2012). Some of the identified compounds within the beans have been used in the treatment of Parkinson's, HIV-related conditions and diabetes (Kovacs, 2007).

2.4 The characteristics of selected legumes

2.4.1 Long bean

The famous long bean in Malaysia is to refer as bora, yardlong bean, asparagus bean, snake bean, long-podded cowpea besides Chinese long bean. It has broad green leaves and white-purplish flower. There are two types of long bean: climbing and dwarf types. This type of legume originated from India and was grown in tropical climate (temperature: 20 – 30 ^{0}C; rainfall: 138 – 168 mm) on large field located in Asia, Central America as well as East Africa. There are five popular long beans known as dark green, light green, black seed, crunchy and white seed. These legumes are commercially grown in Johor, Pahang and also in Perak since 2006. The largest long bean cultivation is in Bentong and followed by Kuantan (Pahang) and Muar (Johor) (Anem, 2011).

This legume fixes approximately 240 kg atmospheric N per hectare and 60 – 70 kg N per hectare are available for rotation of crops in order to maximize the fertility of the soil (Aikins and Afuakwa, 2008). The grain yield of long bean is about 300 kg per hectare (Anem, 2011). Fatokun (2002) reported that long bean is one of the dietary proteins for livestock and human population in the developing world as well as significant food grain for over 200 million people (Abayomi *et al.*, 2008), with over 9.3 million metric tons of annual production (Oritz, 1998). About 20 – 25% of protein can be obtained by consuming valuable legume, for many small holder farmers (Langyintou *et al.*, 2003). They can be grown in marginal land because they provide an excellent source of N in cover crop sequence (Clark, 2007).

2.4.2 Mung bean

Mung bean is originated from the Indian subcontinent and is known as a popular crop in China, India, Bangladesh, Myanmar, USA, Canada, Indonesia, Brazil, Cambodia and Vietnam and has recently been introduced in Australia. This bean has furry pods and parts of each plant with yellow flower. Mung bean is an excellent source of high quality protein, vitamin A, thiamine and iron. Afzal *et al.* (2008) reported that mung bean contains 3% vitamins, 4% mineral, 24 – 26% protein and 51% carbohydrate. Mung bean is not suitable to be grown in high humidity where they can be easily get infected by disease. This crop needs high temperature, low humidity and sufficient moisture in soil (Anem, 2010). It is one of the most important pulse crops in subtropical zones as protein supplements (Mondal *et al.*, 2012).

Mung bean is not well grown in Malaysia but is known as the main producer for bean sprouts in Southeast Asia followed by Myanmar and China (Anem, 2010). It plays an important role in human diet and in maintaining the soil fertility via N_2 fixation (Bhuiyan and Mian, 2007; Mondal *et al.*, 2012). Mung bean can be used as crop for green manure and is ideal as crop rotation after rice planting. This crop is an excellent fodder to the animals as well (Anem, 2010).

2.5 Macronutrients

2.5.1 Nitrogen a major plant nutrient

In agricultural systems, N is known as an imperative factor in plant growth and it acts as a major constituent of chlorophyll, proteins and photosynthetic activity. The earth's atmosphere consists of 78% of N gas and plants are able to use NO_3 as nutrient

(Ontario Ministry of Agriculture and Food, 2005). Nitrogen is commonly obtained by legumes via N cycle and lightning, biologically and from urea, chemically. The average content of N in certain soil is 0.14 % (Griffith, 2004). Ideal conditions such as N availability, soil condition and also field cropping history are required for initiation of BNF which leads to optimum nodulation. Nitrogen is responsible for increasing crop growth, dry matter of crops (Reynolds, 2005), and is an essential component of nucleic acids. Nitrogen exists in organic as well as inorganic form and is required in large amount as metabolic element (Tucker, 2004) and for pastures (Black *et al.*, 2000). The greatest N content can be found in each part of a plant.

Many countries enjoy maximum crop productivity and fertility of the soil by using N fertilizers which may contribute to high expenses of mineral fertilizers and possible harm to the nature (Agamy *et al.*, 2012). Inorganic N fertilizer is significant in giving rise to higher leaf chlorophyll content and growth (Amaliotis, 2004) due to its presence as a constituent element in the protein and chlorophyll molecule structure. Thus, influencing the chlorophyll accumulation in foliage of plants (Tucker, 2004) as well as the leaf colour (Cabrera, 2004). Increase in N concentration in plant is reflected in the gradual increase in protein, sapogenin and chlorophyll content as well as carbohydrate and biomass accumulation in each plant part. Mineral N promotes carbohydrate, chlorophyll and protein content and important nutrient in enhancing the photosynthetic rates, plant height and biomass (Kumar, 2009).

In plant growth and development, N is the key limiting factor because it is involved in the production of essential constituent of proteins and enzymes. It is also involved in metabolic processes such as in the synthesis and transfer of energy, vitamins, nucleic

acids and many other organic molecules, for example, chlorophyll for photosynthesis. Nitrogen deficiency is the main factor that limits bean production in most countries which leads to stunted growth, low quality of leaf and forage crops and yellowish leaves, decrease in leaf area and photosynthetic intensity (Bojović and Marković, 2009). Distribution and uptake of N contributes in crop yield and many aspects of development and growth (Gastal and Lemaire, 2002).

2.5.2 Role of Phosphorus in plants

Phosphorus is known as a genetic "memory unit" of all living things and it is vital as a component of DNA and RNA which linked together by phosphorus linkage (Griffith, 2004). It is involved in development of new cells of plant, where the transfer of genetic code from one cell to another occurs. It is also incorporated into other organic compounds for instance phospholipids, enzymes, phosphoproteins and sugar phosphate. The sugar phosphates then used as building blocks to form storage components and cell structural. This element is also known as the energy unit. It drives the conversion of biochemical reactions in plants as a catalyst. It captures and converts the energy from the sun to form essential plant compounds such as in seed yield, genetic transfer and structure of plant, food formation and forms ATP during photosynthesis.

Phosphorus is important in the growth of seedling, root development, seed productions, flower formation, stem and stalk strength, crop quality and also improves the ability to withstand unfavorable environment as well as resistance to plant diseases. By triggering the formation of root, it enhances the ability of plants to uptake

water and other nutrients (Griffith, 2004; Sandra, 2011). The root extracts nutrients which have very low concentrations from the soil solution. The movement of nutrients within the plant requires high energy P compounds to counter the forces of osmosis. It increases the N – fixing of legumes by initiating the root nodulation. Availability of P in plant increases yield of both perennial and annual legumes (Moir *et al.*, 2012).

The amount of P is low on the surface of soil (0.60 %) (Griffith, 2004) and it can be obtained from rock phosphate. This vital nutrient is required in large amount for rapid growth especially annual plants such as legumes while plants grown in warm climates required the least amount. Roots can absorb P mostly in the form of primary orthophosphate ion (H_2PO_4) rather than secondary orthophosphate (HPO_4) (Sandra, 2011). This is because the latter form may increase the soil pH.

The deficit of phosphorus can be observed based on the abnormal discoloration, seed development, stunted growth of plant, crop maturity and decrement in leaf surface area and number of leaves. The discoloration can occur on leaves (dark bluish – green) and stem (purplish) due to the accumulation of sugars from the synthesis of anthocyanin in the leaves (Griffith, 2004). Inadequacy of P may also lead to reduction in the number of seed, seed viability and size. Phosphorus contributes to the development of flower and fruit as well as plant growth and root system. Initiation of nodules development as well as efficiency of the symbiosis relationship between rhizobium and legumes is influenced by P (Nyoki and Ndakidemi, 2014).

2.5.3 Potassium as a plant macronutrient

Potassium is one of the key nutrients where its average soil content (0.83 %) is higher than that of P and N (Griffith, 2004). Potassium is required to promote the growth of a plant (especially plants with high carbohydrate such as potatoes), increase the length of stem, quality and the size of vegetables, fruits and grains as well as enhance the green colour of leaves. This seventh most abundant element within the crust of Earth can be obtained naturally by the evaporation of water from natural salt lakes, like the Dead Sea, and chemically through potassium fertilizer. Crops may absorb K in the form of dissolvable K^+ ion. It is known as "the regulator" (Sandra, 2011) and acts as a catalyst in many enzymatic processes that are essential for plant growth and is involved in the regulation of water within the plant which is known as osmoregulation. This process regulates the closing and opening of the stomata that affects the uptake of CO_2 for photosynthesis and transpiration cooling. Water transport in the xylem, cell elongation for growth and cell turgor pressure are affected by the osmoregulation process (McAfee, 2011). This element tolerates to unfavourable environments by resisting drought and other extreme temperatures and increases resistance of crops to pests and diseases and ensures that all the transport systems within the plants function efficiently (McAfee, 2011; Sandra, 2011).

The synthesis of proteins and root and stem growth are influence by inorganic K. Potassium also contributes in yield of plant and chlorophyll concentration in leaves (Chapagain and Wiesman, 2004). It assists in the relation between legumes and rhizobia and ensuring the process is effective. Potassium has a big impact on BNF, yield of crops and N accumulation in plants as a result of increase in accumulation of

carbohydrate in plants, root numbers, nodule size and productivity other than nodule number (Johnston, 2006). NPK fertilizer has been used commercially to promote maximum chlorophyll biosynthesis, growth characters, crop yield and yield components (Abd El-Aziz, 2007; Agamy *et al.*, 2012) as well as plant biochemical status (Kumar, 2009). The highest rate of uptake of K on soil can be achieved when it possesses slightly acidic to neutral pH and in addition of moist, well aerated and warm. Increase in soil temperature may lead to increase in root activity and growth as well as metabolic activities within the plant. The diffusion rate of K in the soil solution increases where uptake of K by the root system increases too (Armstrong *et al.*, 1998). The uptake of K may decrease due to excess soil moisture that can cause a decrease in levels of soil oxygen and respiration rate of root system of plants. The amount of leaching of K increases when there is excess water especially in sandy soil. The displacement of K from the exchange sites on the soil particles and for uptake by the plants root system occurs when availability of other cations, such as Na^{+}, Mg^{2+} and Ca^{2+}, increases particularly in alkaline soil. Thus, the availability of K within soil is affected by the presence of these cations where they compete with each other to be taken up by plant roots (McAfee, 2011). Inadequate of K may cause yellowing of leaves at the lower leaf blades, scorching of leaf margins as well as dieback of the leaf tip (Sawyer, 2004).

2.6 Physiological and biochemical parameters

2.6.1 Dry matter accumulation and crop yield

The analysis of dry matter accumulation in beans is important to assess the growth rates throughout the crop cycle and predicting yield. It shows the variations in the nutrients uptake and application of different dose of fertilizers (Silva *et al.*, 2002) towards the crop. Dry matter accumulation is one of the important factors in mineral elements determination required by photosynthesis (Delden, 2001). Dry matter accumulation and pod production may vary depend on relative growth rate (RGR) and crop growth rate (CGR), leaf area (LA) and net assimilation rate (NAR). Some physiological traits reflect the yield components and essential in quantifying the growth components and crop improvement (Mondal *et al.*, 2012).

Biomass production of legumes are closely related to the amount of N_2 fixed (Unkovich *et al.*, 2011). The yield of crop can be estimated by the nutrients supplied which relative to the required levels in order to achieve optimal yield. The amount of N in shoot is related closely to the amount of production of biomass, about 20 – 25 kg of N. Increases in legume growth and biomass as a result of increased in fixation of N where percentage of N and dry matter accumulation enhanced (Hayes *et al.*, 2008). Accumulation and distribution of dry matter influence the crop yield (Wang *et al.*, 2012) and plant productivity (Zhu *et al.*, 2011). It is vital in building high grain yield and biomass which related to N cycling (Epstein and Bloom, 2005).

2.6.2 Chlorophyll content

Chlorophyll content of leaf tissue is a good indicator of photosynthetic activity (Chowdhury and Kohri, 2003) and is important in managing chemical and fertilizer application (Haboudane *et al.*, 2002; Wu *et al.*, 2008). Chlorophyll content is well-known as a measurement of plants physiological activities and an index of growth of plant as well as production of organic matter (Lahai *et al.*, 2003). Chlorophylls, carotenoids and xantophylls are the main group of photosynthetic pigments. The photosynthetic pigments are mostly soluble in non-polar solvents such as acetone, chloroform and ether as a consequence of insoluble in water. Generally, chlorophyll *a* is the main photosynthetic pigment (Campbell and Reece, 2005) and chlorophyll *b* known as an accessory pigment in plants that absorb specific wavelengths of light transfer energy to the chlorophylls.

Chlorophyll *a* is a primary pigment in all plant species while chlorophyll *b* is less abundant. Both chlorophyll *a* and *b* possess similar structures but different R group on the porphyrin group (R = CHO – aldehyde; CH_3 – methyl, respectively). Chlorophyll *a* absorbs light with wavelengths of 430 nm and 662 nm and appears green due to strong green light reflection and most abundant pigment in photosynthetic organisms that produce oxygen. Chlorophyll *b* substitutes for chlorophyll *a* in photosystem II (Xu *et al.*, 2001) and it helps to enhance the efficiency of photosynthesis process in green plants by increasing the portion of visible spectrum that is absorbed as energy. Chlorophyll *b* absorbs maximum light of 453 nm as well as 642 nm (Campbell and Reece, 2005).

Chlorophyll is usually abundant in leafy vegetables. There are about half a million chloroplasts per square millimeter of leaf surface. Healthy plants with large amount of chlorophyll are expected to have maximum growth. Chlorophyll molecule is the light energy receptor that contains N. Inadequate of N will lead to the suppression of chlorophyll formation since this nutrient is an essential component in the chlorophyll molecule to carry out photosynthesis (Campbell and Reece, 2005; Wallace, 2008).

2.6.3 Photosynthesis

Photosynthesis is a process where chlorophyll in the chloroplasts harvest light energy from the sun and convert it to chemical energy stored in sugar and other organic molecules. Photosynthesis mainly occurs in the mesophyll cells in the chloroplast. The chloroplast has an outer and inner membrane besides grana (stacks of thylakoid disks) which are connected by intergranal lamellae membranes. Stroma in the chloroplast lies within the inner membrane and outside the thylakoid membrane. Oxygen production and light trapping occur in the thylakoid disks (light – dependent reactions) whilst the light – independent reactions (dark reactions) take place in the stroma where CO_2 fixed to carbohydrates (Alberts *et al.,* 2002). The rate of plant growth depends on the rate of photosynthesis and leaf is an important organ in determining the photosynthetic productivity (Bojović and Marković, 2007) which relies on development of leaf area, leaf age and light intercepted by the leaves.

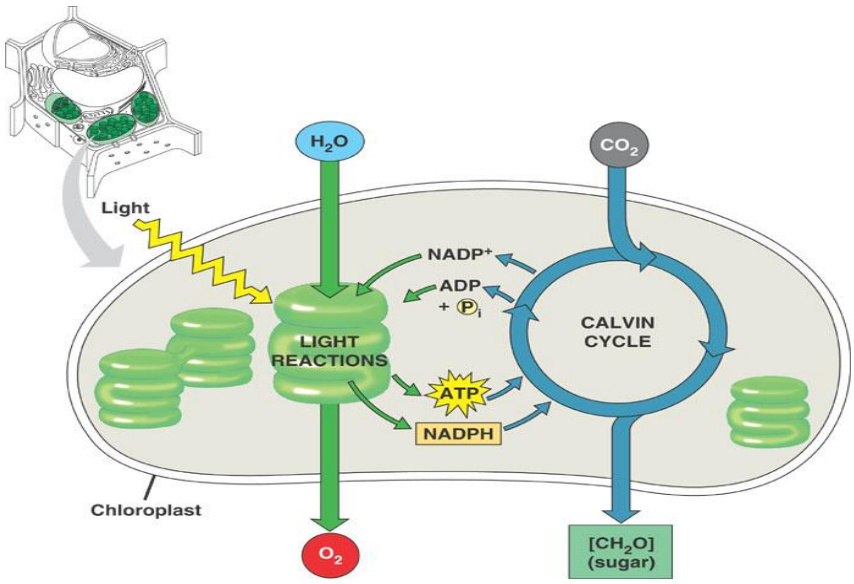

Figure 2.2: Photosynthesis (Campbell and Reece, 2005).

About 90% of the total dry matter accumulation contributes by photosynthesis and efficient mechanism of photosynthesis by plant lead to higher crop production biologically (Biswas *et al.,* 2001). Development of crop yield is related directly to photosynthesis, partitioning and also translocation (Neto, 1991). Strong positive correlation can be observed between leaf N content and photosynthesis due to increase in leaf N content. Limitation of N may cause reduction of chlorophyll content and RUBISCO activity which lead to decrease in photosynthesis (Cechin and Fumis, 2004).

2.6.4 Root nodules

Nodules form symbiotic relationship with *Rhizobia* in order to fix N via N_2 fixation mechanism. Nodules of legumes are rich in Cys cluster proteins (CCPs) that possess antimicrobial activity and play a role in protecting nodules from pathogens (Samac and Graham, 2007). Nitrogen-fixing bacteria are attracted to roots of legume plants

when flavonoids released from the plant root hairs and the rhizobia induce the lipochitooligosaccharide Nod factors synthesis. The infection of root hairs of the legume plants is mainly caused by symbioses with proteobacteria. Once in contact, the root hairs bind with the attracted bacteria. Bacteria cells release a chemical and invade the root hairs that results in curling and proliferation in infected area and induce the growth of infection thread into the zone of meristematic cells of root cortex. Hence, a plant derived symbiosome membrane surrounded the bacteria which released from the infection thread of plant root hairs by endocytosis (Gage, 2004).

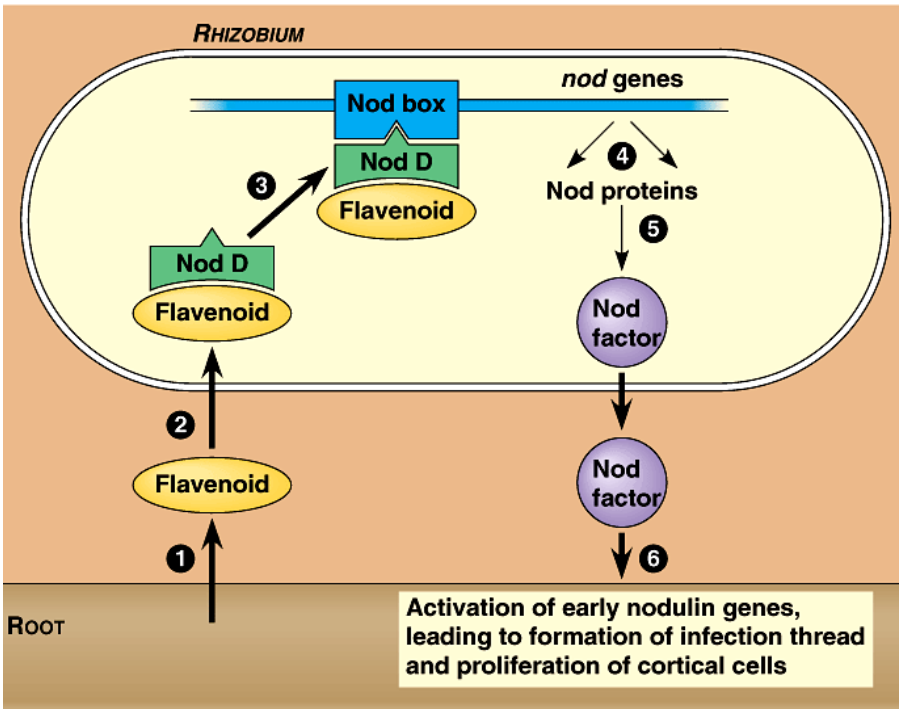

Figure 2.3: Formation of root nodules (Campbell and Reece, 2005)

The bacteria are permitted to occupy the cells interior which results in development of root structures known as nodules (Oldroyd and Downie, 2008). The invasion of bacteria caused the root hair curled which leads to thread formation in root hair of legumes. The invaded bacteria then multiplied in order to form nodules where N

fixation takes place. Nodules or "bumps" occupied with thousands of symbiosomes were developed (Prell *et al*., 2010). The bacteria invaded the host plant uptake atmospheric N gas from soil and air and converts to ammonia in the presence of nitrogenase enzyme. The bacteria then differentiate into bacteroids within the nodules and nitrogenase enzyme complex catalyzes the conversion of atmospheric N to other form of N that can be absorbed easily by plants, ammonia (NH_3) (Hirsch, 2009; Ferguson *et al.*, 2010) and is essential in crop yield and plant growth. In this interaction, rhizobia provide atmospheric N to the legumes and then protein-rich seeds which are important in human and animal diets are produced and 200 millions of N kg ha^{-1} annually (Samac and Graham, 2007; Ferguson *et al,* 2010). The N fixing leguminous trees nodulated with effective rhizobia and are able to act as a promising alternative to fertilizer in plant productivity in developing countries (Degefu *et al.*, 2011).

2.6.5 Nitrate reductase activity (NRA)

Nitrate reductase is known as substrate-inducible enzyme and rising with nitrate concentration supplied. The nitrate reductase is comprised of two identical subunits where each subunit possessed and electron transport chain (ETC) with the presence of one heme of cytochrome-*b* type, one flavin adenine dinucleotide molecule (FAD) and one molybdenum cofactor (MoCo). The molecular of each subunit differ from 99 to 104 kDa. The MoCo is a cofactor where Mo attached by two S bonds and it has pterin with side chain. The oxidation states of bound Mo atom may change from +IV to +VI (Heldt and Piechulla, 2011). This Fe-S Mo flavoprotein (EC 1.7.1.1) is referred as

NADH:nitrate oxidoreductase (McDonald, 2014). It is the key enzyme involves in the NO_3 assimilation process where plants uptake NO_3 as source of N. Nitrate reductase is mainly a cytosolic enzyme that is present in leaves as well as roots of higher plants. This enzyme also can be found in plasma membrane and regulated at the post-translational and transcriptional levels and is under the control of water potential, N source, light, temperature, CO_2, pH, phytohormones and O_2 (Franková *et al.,* 2005). NR catalyzes the conversion of nitrate ion (NO_3^-) to nitrite ion (NO_2^-) as follows:

$$NO_3^- + NAD(P)H + H^+ \longrightarrow NO_2^- + NAD(P)^+ + H_2O$$

The NO_2 produced is then reduced to ammonium ion (NH_4^+) with the help of nitrite reductase (NiR). At higher concentrations, both NO_2 and NH_4 are toxic. A combination of control NR catalytic activity, NR protein degradation and NR expression provide a rigid control of the NO_2 concentration. NADH or NADPH is generally used as a reductant in NO_3 reduction. This enzyme is up-regulated in the presence of nitrate, light, high concentration of CO_2 and photosynthetic production (sucrose). Several lines of evidence have demonstrated that the sequential of reduction of NO_3 to NO_2 via NR along with mitochondrial ETC in the plant roots contributed energy supply under microaerobic conditions (Igamberdiery and Hill, 2009). Nitrate reductase activity in plants is often correlated with crop growth and plant yield. It also can estimate the N status of a plant (Srivastava, 1980).

2.7 Impacts of fertilizer on the nodule formation in legumes

Native rhizobia, soil N and inoculants of rhizobia are three sources where legume can obtain N. Even though legumes can fix N_2, they also obtain some of their N from the soil. High N requirements of legumes can be satisfied by N_2 fixation because the

conversion of N_2 to NH_3 takes place inside the plant. Legumes can improve N supply for succeeding crops because of their remarkable capability in fixing large amount of N. Fertilization is a less efficient way to provide N to legumes. This is because some of the fertilizer can be permanently or temporarily lost. Legume plants may require a lot of energy to move inorganic N from the soil via the cell membranes into the roots. More energy is required once the N is inside the plant in order to convert N_2 to NO_3 or NH_3. Application of fertilizer may increase acidity of soil which leads to inhibition of formation of nodule. Fertilizers may suppress the normal formation of nodules. It also may eliminate the detrimental effect of seed. Certain salts can decrease the formation of root hairs and as a result it decreases nodule formation which is important for N_2 fixation. High concentration of fertilizer causes a desiccation of the soil which also can inhibit the action of nodule bacteria. Nitrogen fixation is reduced with an increase of levels of N fertilizer. Biederbeck *et al.,* (2013) reported that high availability of inorganic N in the soil may reduce the amount of N fixed by the legumes greatly.

2.8 Impacts of fertilizer on environment, human health and society

Agriculture in the beginning of human history was sustainable where synthetic chemicals were not used. When chemical agriculture was established in the late 1940, the soil biomass and its natural balance were disrupted as well as plants destruction by invasion of pests. In modern agriculture, huge pressure has mounted on Mother Nature where man relies on chemical fertilizers and pesticides to increase the crops production (Plimmer, 1984). The usage of NPK fertilizer can have negative

sociological impacts as well as ecological problems for instance, ozone depletion due to the release of nitrous oxide (NO_2) by denitrification process (decomposition of N fertilizer) and greenhouse effect or global warming as a result of liberation of carbon dioxide (CO_2) during combustion of fossil fuel. Active NO_2 is more vigorous in contribution to greenhouse impact compared to CO_2 (Crutzen *et al.*, 2008).

High application of N fertilizers result in the leaching out of nitrate ion into the groundwater that caused eutrophication (cloudy or discoloured water) of waterways (Graham and Vance, 2003), soil acidification by emission of ammonia gas (NH_3), accelerate algae growth as well as disrupt the water ecosystems. Optimization of inorganic N inputs and reliance on chemical fertilizers are required to prevent pest problems too. Excessive supply of N can cause less vigorous plant growth due to the fact that higher concentration of N ion in the soil may reduce the plant uptake of other essential nutrients (Kumar, 2009).

Nitrogen fertilizer application gives rise to environmental problems (Lawlor *et al.*, 2001) especially related to NO_3 loss in the environment. When N input exceeds the demand, the source of N that builds up in the soil is mostly NO_3. In general, only a little amount of the NO_3 will be taken up by the roots. Accumulation of NO_3 in plants arises from the uptake of nitrate ion (NO_3^-) in excess of its reduction and subsequent assimilation in the metabolism. The excess NO_3 in plants immediately accumulated in shoots and roots, mostly in vacuoles of both shoots and roots. Anjana *et al* (2006) reported that accumulation of NO_3 in plants is influenced by certain factors and varies widely between species. Usually, about 72 – 94% of the total daily NO_3 intake contributed from leafy vegetables that are consumed by people every day (Eicholzer

and Gutzwiller, 1998). Unfortunately, leafy vegetables maximally contribute to NO_3 consumption in human diet beyond safe limits (Anjana *et al*., 2007). This is revealed by Anjana *et al.,* (2006) that a significant number of spinach and chenopodium collected from the local markets of Delhi contained higher concentration of NO_3 compared to the Acceptable Daily Intake (ADI) limit e.g. an average 60 kg person consumed 100 g of vegetables per day. High intake of NO_3^- (N – nitroso compounds) by human may increase the risk of various health related problems, especially esophageal and nasopharyngeal cancer (Eicholzer and Gutzwiller, 1998).

Most pregnant women consumed green leafy vegetables as a main source of iron, vitamin, nutritional and therapeutic properties (Santamaria, 2006). But, these vegetables may contain high content of NO_3 when treated with high amount of N fertilizer which cause pregnancy failure and may contribute to various diseases, example stomach cancer and Non-Hodgkin's Lymphoma. Therefore, little amount of NO_3 in leafy vegetables may reduce the risk of human illness (Luo *et al.,* 2006). The N fertilizers cause environmental problems associated with leaching into our water systems and production of greenhouse gas. To reduce the usage of the fertilizers, alternative ways to increase N availability to plants are investigated. Nodulation process forming of symbiosis between N-fixation bacteria and plant is one of the interested studies nowadays.

These days, the use of bio-organic fertilizers in agriculture caught many attentions due to the usage of chemical fertilizers and their negative consequences experienced by the environment. Bio-organic fertilizers are formed by inoculation of compost from organic material which undergone rapid decomposition by introducing homogenous

microbial inoculants (Philippine Coconut Authority, 2004). These fertilizers increase water holding capacity, prevent nutrient leaching and add more mineral nutrients to the poor sandy loam soil (Agamy *et al.*, 2012). Thus, improve the physical and chemical properties of soil and soil characteristics (Moller, 2009). Khaliq *et al.*, (2006) emphasized that new production of technologies which sustain ecology and economy are required to overcome the environmental impacts and high cost aspect regarding the usage of mineral N.

Humic substances are also recognized by most soil scientists and agronomics as the most important component a healthy fertile soil. Man need to realize the importance of humic substances and their values as fertilizer ingredients to support plant growth has never been more important than it is today (Pettit, 2012). These substances are the end product of decaying organic matter which formed via humification. They contribute the black colour of soil surface and affect chemical and physical properties of soil and improve soil fecundity (International Humic Substances Society, 2007).

The application of NPK fertilizer can reduce the N_2 fixation efficiency by poor production of nodules and NRA that is required to maintain soil fertility. Nitrogen fertilizer interrupts the N_2 fixation process due to NO_3 accumulation within the plants which inhibits the conversion of N_2 to NH_3. Application of NPK fertilizer is not necessary to be used to grow plants on fertile soil. The usage of highly cost inorganic N fertilizer might cause environmental and health problems and it needs to be halt.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental site and management

The study was conducted in a Greenhouse at the Institute of Biological Sciences, Faculty of Science, University of Malaya (UM), Kuala Lumpur (3°7'25"N, 101°39'11"E), Malaysia during November 2011 to December 2012. Long bean (dwarf type) and mung bean were tested in this study. Legume seeds were collected from Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor. The soil of the experimental pot was black clay loam with a pH of 6.14. Plastic pot (0.25 m^2) was used to conduct the experiment and each pot was filled with 5 kg of soil.

Dwarf long bean and mung bean were grown without NPK fertilizer (0F) and with NPK fertilizer at the rate of 2 – 4 – 2 g/m^2. Urea (46% N), triple super phosphate (46% P) and muriate of potash (60% K) were used as the sources of NPK fertilizer. Each legume plant was tested in an individual experiment. The experiment was conducted under completely randomized design (CRD) with six replications. Seeds were soaked with water for 1 hour and then planted in pots as per treatment schedule. Nitrogen fertilizer was applied into each pot at 7 days after emergence (DAE) of each legume while P and K were applied before planting the legumes. There were four legume plants raised in each pot.

3.2 Soil analysis

Soil samples were taken before planting and after harvesting of long bean and mung bean grown with and without fertilizer of each replication. Collected soils were dried in an oven at 72 ^{0}C for 48 hours (Memmert, Interscience) (Appendix 3). Soil samples were analyzed using CHN (Carbon, Hydrogen, Nitrogen) analyzer (Perkin Elmer 2400 Series II) at the Department of Chemistry, Faculty of Science, University of Malaya to determine N content in the soil samples. The samples were ground into very fine powder using mortar and pestle. Empty tin capsules were used as blank values. About 2 to 3 mg of soil samples were weighed in tin capsules and folded. The samples were then placed in the autosampler and analyzed by CHN analyzer (Perkin Elmer 2400 Series II) (Appendix 3).

3.3 Chlorophyll content

Chlorophyll content was estimated by Arnon's method (Arnon, 1949) and using the SPAD meter.

3.3.1 Standard method (Arnon's method)

Six fully expanded leaves were selected randomly at vegetative, flowering, pod formation and maturity stages to determine the chlorophyll contents using Arnon's method (Arnon, 1949). Approximately 50 mg leaf tissue was homogenized in 10 mL of 80% acetone (R&M Chemicals) (Appendix 1) using mortar and pestle under low light. The suspension was decanted through filter funnel (Buchner) using Whatman filter paper No. 1. The residue in the mortar was washed with the solvent and filtered. The chlorophyll content was measured at 645 nm and 663 nm absorbance by using

Thermo Scientific Genesys 10S UV VIS spectrophotometer (Appendix 3). The chlorophyll concentration was calculated then using specific absorption coefficients for total chlorophyll, chlorophyll *a* and *b* provided by Arnon (Arnon, 1949; Makeen *et* al, 2007; Wu *et al*, 2008). The data were then recorded as follow:

Total chlorophyll (mg/g) $= 20.2 (Abs_{645}) + 8.02 (Abs_{663})$

Chlorophyll *a* (mg/g) $= 12.7 (Abs_{663}) – 2.69 (Abs_{645})$

Chlorophyll *b* (mg/g) $= 22.9 (Abs_{645}) – 4.68 (Abs_{663})$

Where, Abs_{645} and Abs_{663} = Absorbance at 645 nm and 663 nm wavelength, respectively.

3.3.2 SPAD meter

Portable SPAD meters, was utilized to determine chlorophyll concentration other than destructive method and interpreted the nutritional status of plants. This technique is possible to be applied in larger field studies. The relationship between readings from chlorophyll meter and chlorophyll content is non-linear but the readings were correlated with the photosynthetic rates of leaves (Uddling *et al.,* 2007). A portable chlorophyll meter [SPAD-502, Soil-Plant Analysis Development (SPAD) Section Minolta Camera Co., Osaka, Japan] (Appendix 3) was used to estimate chlorophyll content from randomly selected six fully expanded leaves per plant at vegetative, flowering, pod formation and maturity stages. The readings were taken from the same marked plants at each stage and were analyzed statistically.

3.4 Photosynthetic rate

A portable Lci-SD (Leaf Chamber/Soil Respiration Analysis System) (Thermoscientific) (Appendix 3) was used to measure photosynthetic active radiation (PAR) of both legume plants at pre-flowering, initiation of flowering, pod formation and maturity stages. Photosynthetic rate was measured by the Lci – SD. Three readings were recorded from randomly six fully expanded leaves of long bean and mung bean legume plants once they were placed at the leaf chamber. The readings were taken at 11.00 a.m. from the same marked plants.

3.5 Dry matter and yield attributes

3.5.1 Total dry matter

Six individual plants of each crop were excavated at vegetative, flowering, pod formation and maturity stages. Leaf, stem, pods, and flowers of each plant were separated and oven dried at 72 ^{0}C for 48 hours (Memmert, Interscience) (Appendix 3). The dry matters of the plant parts were weighed using analytical balance (AND FX-3000) (Appendix 3). The data were recorded.

3.5.2 Yield and yield attributes

Number of pods and seeds per pod were counted from six individuals at maturity stage of each legume plants. Then, grain was separated from pods. The weights of 100-seed were weighed using analytical balance (AND FX-3000) (Appendix 3). Grain yield of each legume plant was measured at 10 – 12% moisture and recorded.

3.6 Nodule collection

3.6.1 Number of nodules and nodule dry weight

Nodules were collected from randomly selected six plants of both legumes at vegetative, flowering, pod formation and maturity stages. The excavated roots and nodules were washed under running tap water to remove soil and inert particles. Exposed root nodules were collected from the root systems using forceps and amassed. The number of collected nodules of each legume plants were counted and then dried in an oven at 72 ^{0}C for 48 hours (Memmert, Interscience) (Appendix 3) to determine nodule dry weight per plant (Appendix 4).

3.6.2 Preservation of nodule

Some of the detached nodules were preserved in vials containing blue dessicated silica gel at 4 ^{0}C and kept for NRA estimation. Nitrate reductase activity was determined to see the effect of NPK fertilizer on NRA in root nodules (Appendix 3).

3.7 Determination of nitrate reductase activity (NRA)

The nitrate standard curve was used to estimate NRA (Appendix 3). Nitrate reductase activity was determined from the collected nodules at vegetative, flowering, pod formation and maturity stages of each legume plant. Based on Sym's method (1984), about 0.02 g of fresh nodules were weighed and homogenized in 5 mL of 0.01M phosphate buffer (pH 7.0) containing 20 mM KNO_3 (R&M Chemicals) (Appendix 1). One set of samples were placed at test tubes labeled as T_0 and another one set of samples were placed at test tubes labeled as T_1. One mL of assay medium was pipetted

into each test tube and the tubes were covered with parafilm. T_1 test tubes were incubated in water bath for 1 hour at 30^0C while T_0 was placed in a beaker containing boiling water for 5 minutes and then cooled at room temperature. T_1 test tubes were taken out from the water bath and placed in the beaker containing boiling water for 5 minutes. The T_1 test tubes were removed and left at room temperature to cool. About 0.5 mL of 1 % sulfanilamide solution (SA) (Appendix 1) and 0.5 mL of 0.02% N-1-Naphthylethylenediamine dihydrochloride solution (NEED) (R&M Chemicals) (Appendix 1) were added into each reaction medium. Pink diazo colour complex was developed after few seconds due to NO_2 formation. The absorbance was read at 540 nm (A_{540}) and test tube containing phosphate buffer (blank) treated with SA and NEED was used to re-zero the spectrophotometer (Thermoscientific) (Appendix 3). There were six replications of NR extracts used. The amount of NRA in each growing stage was calculated as micromoles NO_2 produced per hour per g fresh weight (NO_2^- h^{-1} $gfwt^{-1}$):

$$\mu\text{mols } NO_2^- \ h^{-1} \ gfwt^{-1} = \frac{\mu\text{mols } NO_2^- (t = 1) - \mu\text{mols } NO_2^- (t = 0)}{0.02 \text{ g plant parts}}$$

(Fernando, 1992)

3.8 Statistical analysis

Data on chlorophyll content, photosynthetic rate, dry weight of plant parts, number of pods, seeds per pod and seed yield per plant, dry weight of nodules and NRA of nodules were analyzed statistically using Microsoft Excel 2010. Treatment means of all attributes were compared by using Fishers Least Significant Difference (LSD) test and t-test at a 0.05 probability level.

CHAPTER 4

RESULTS

4.1 Soil N

Initial soil N status was similar for both long bean and mung bean. Soil N status significantly improved after harvesting of both crops especially long bean grown without fertilizer and mung bean grown with NPK fertilizer (Table 4.1).

Table 4.1. The nitrogen content in soil before planting and after harvesting of long Bean and mung bean

Legumes	**Nitrogen in soil (mg kg^{-1})**	
	Before planting	**After harvesting**
Long bean		
Control	70.5 ± 4.30	134.5^{b} ± 1.15
Fertilizer	71.0 ± 7.25	160.3^{a} ± 4.20
Mung bean		
Control	71.0 ± 7.25	93.5^{b} ± 1.25
Fertilizer	70.5 ± 1.25	184.5^{a} ± 4.15

Means followed by the same letters are not significantly different for each treatment means ($P < 0.05$) by LSD, ns = non-significant

*Both long bean and mung bean grown with fertilizer were harvested at 48 DAE and 64 DAE, respectively.
*Both long bean and mung bean grown without fertilizer were harvested at 54 DAE and 67 DAE, respectively.

4.2 Chlorophyll content

4.2.1 Estimation of chlorophyll content using Arnon's method

Total chlorophyll, chlorophyll *a*, chlorophyll *b* and chlorophyll *a/b* ratio of both legumes were appreciably affected by the treatment variables. The highest chlorophyll contents were found in long bean (2.99 mg/g) and mung bean (2.97 mg/g) grown with fertilizer at the onset of flowering and thereafter declined until maturity (Figure 4.1) (Appendix 2).

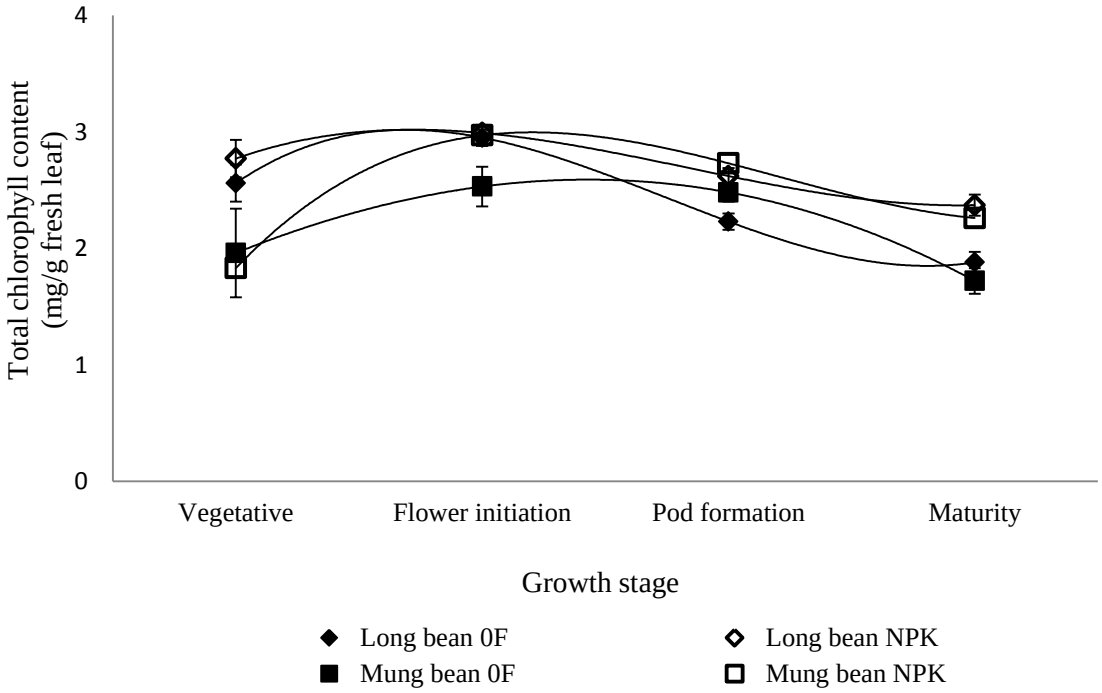

Figure 4.1. Total chlorophyll content of Long bean and Mung bean over time at different growth stages.
Vertical bars represent LSD at $p<0.05$ among the treatments

The highest chlorophyll *a* (2.25 mg/g) content was recorded in long bean grown with fertilizer at flower initiation stage and then declined over time (Figure 4.2). Mung bean

grown without fertilizer obtained the least chlorophyll *a* (1.73 mg/g) but its chlorophyll *b* (0.80 mg/g) reached peak at flower initiation stage (Figure 4.3). The chlorophyll *a/b* ratio of mung bean grown without fertilizer (0.62) was higher at pod formation stage (Figure 4.4) (Appendix 2).

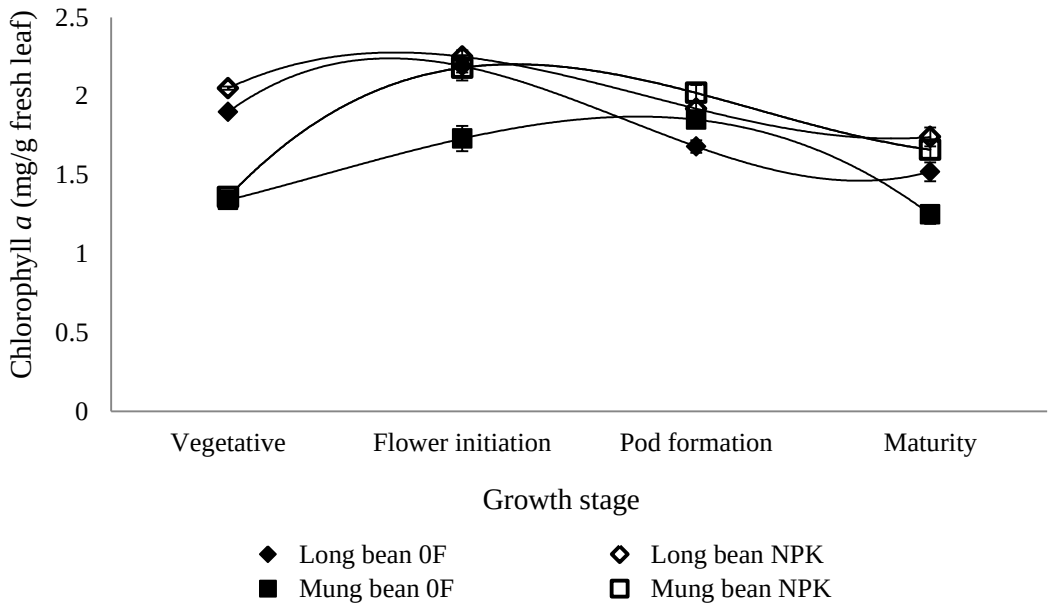

Figure 4.2. Chlorophyll *a* content of Long bean and Mung bean at different growth stages.
Vertical bars represent LSD at $p<0.05$ among the treatments

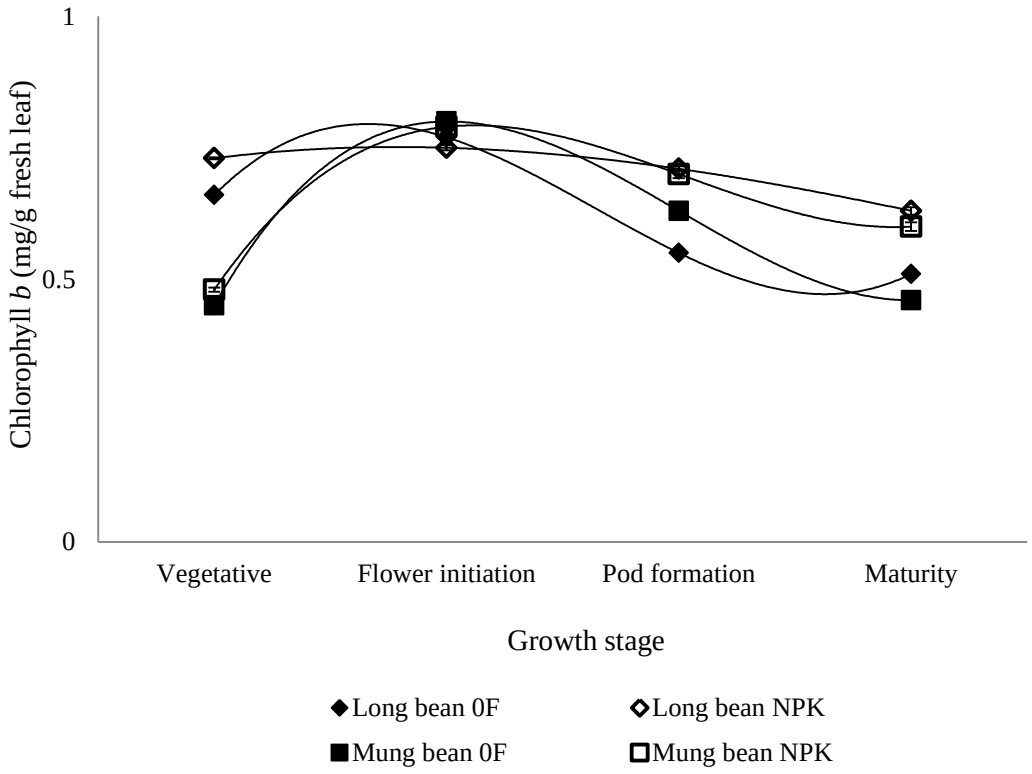

Figure 4.3. Chlorophyll *b* content of Long bean and Mung bean at different growth stages.
Vertical bars represent LSD at $p<0.05$ among the treatments

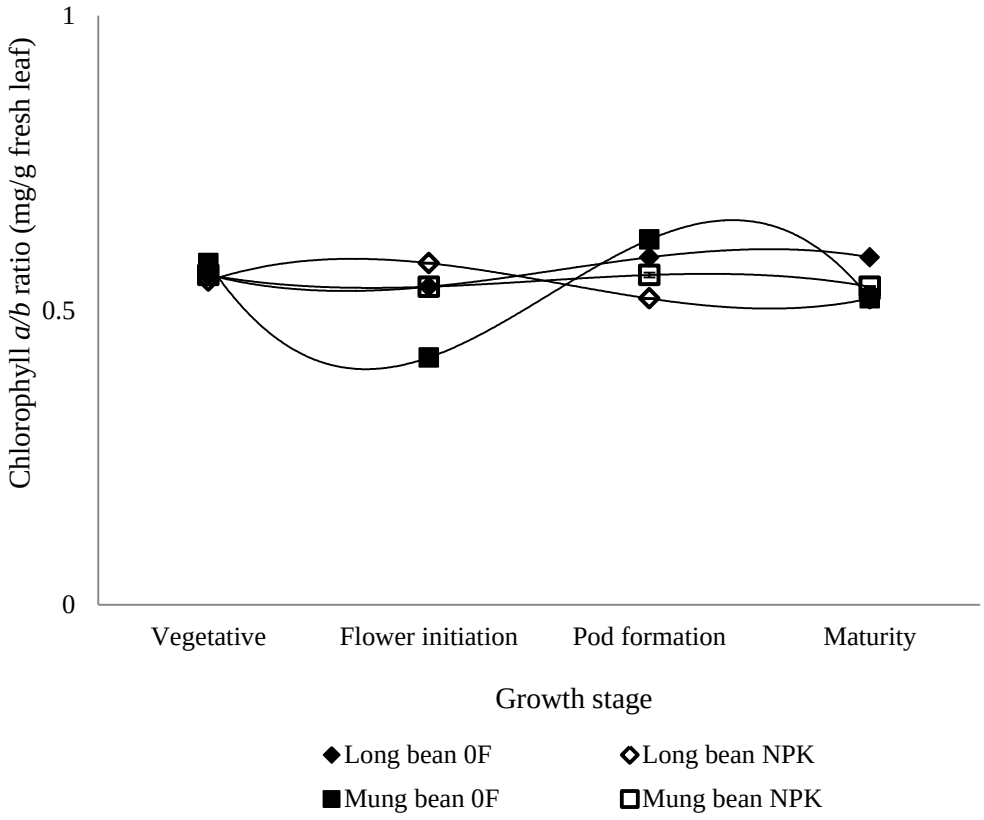

Figure 4.4. Chlorophyll *a/b* ratio of Long bean and Mung bean at different growth stages.
Vertical bars represent LSD at $p<0.05$ among the treatments

4.2.2 Estimation of chlorophyll content by SPAD meter

Leaf SPAD values of both legumes were appreciably affected by application of NPK fertilizer. Long bean grown with fertilizer showed the highest SPAD value (41.35) at flowering stage. The leaf SPAD values were higher in long bean and mung bean grown with fertilizer than both beans grown without fertilizer. Both legumes showed increase of leaf SPAD values at vegetative stage and reached peak at flower initiation stage thereafter declined gradually (Figure 4.5) (Appendix 2).

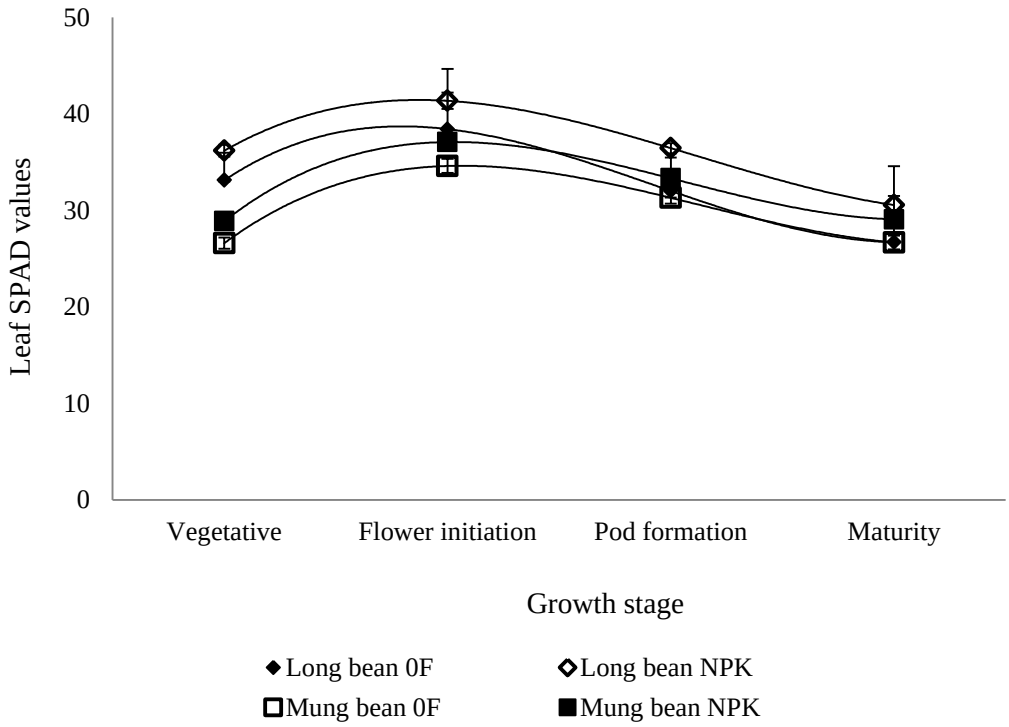

Figure 4.5. Leaf SPAD values of Long bean and Mung bean at different growth stages.
Vertical bars represent LSD at $p<0.05$ among the treatments

4.3 Rate of photosynthesis

Leaf photosynthetic rate was appreciably influenced by application of NPK fertilizer throughout the crop growing period. The highest photosynthetic rates of both legumes were recorded at the onset of flowering and thereafter decreased gradually up to the end of crop growth. Long bean grown with fertilizer showed higher photosynthetic rate (1.26 µmol m^{-2} s^{-1}) than mung bean grown with fertilizer (0.97 µmol m^{-2} s^{-1}). Both long bean and mung bean grown without fertilizer also recorded better photosynthetic rate at flower initiation stage (Figure 4.6) (Appendix 2).

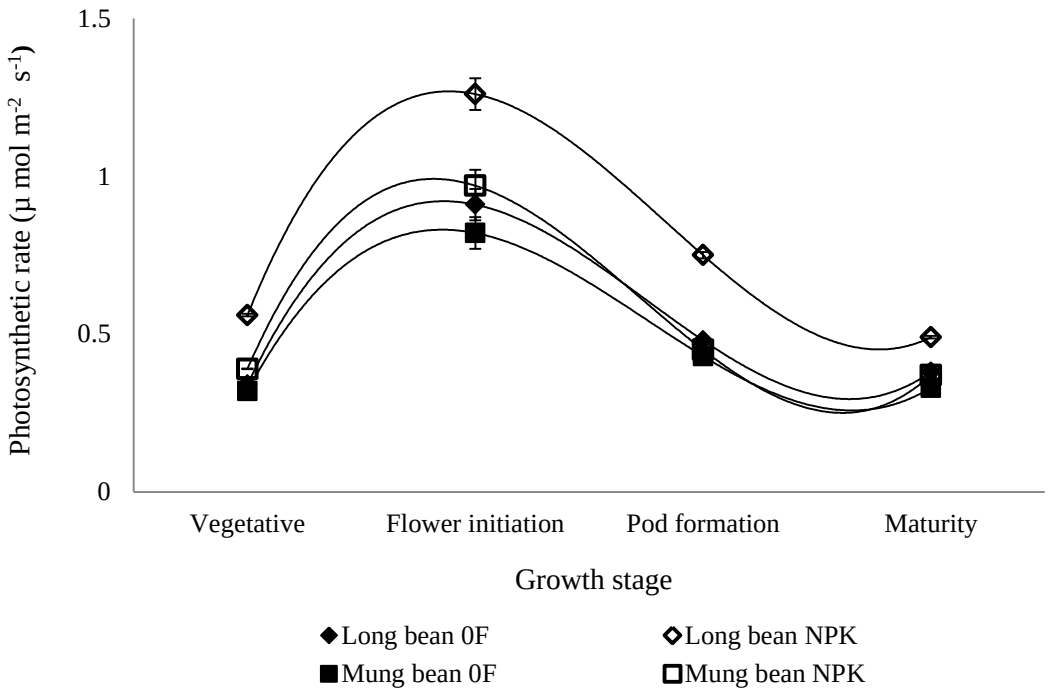

Figure 4.6. Photosynthetic rate of Long bean and Mung bean at different growth stages.
Vertical bars represent LSD at $p<0.05$ among the treatments

4.4 Number and dry weight of nodules

The application of fertilizer gave an inhibitory effect on number of nodules and dry weight of nodules. Both legumes grown without fertilizer produced maximum nodules at flower initiation stage and declined with increase of plant age. Long bean grown without fertilizer produced the highest number of nodules (17.33/plant) at flower initiation stage followed by mung bean grown without fertilizer (15.50/plant) at 43 DAE (Figure 4.7) (Appendix 2).

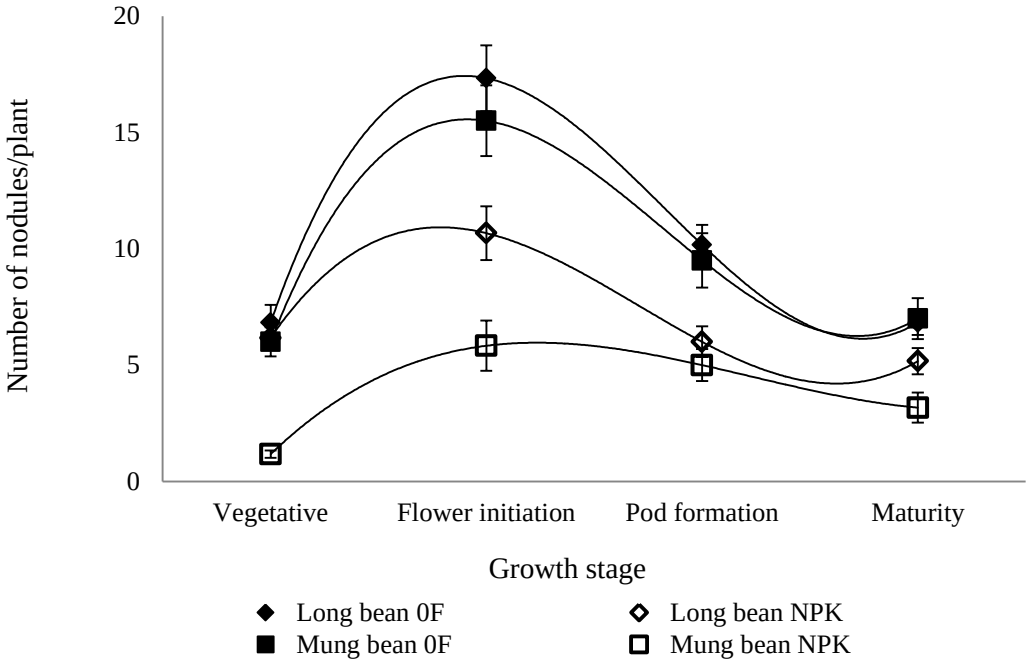

Figure 4.7. Number of nodules per plant of Long bean and Mung bean at different growth stages.
Vertical bars represent LSD at $p<0.05$ among the treatments

Similarly, dry weight of nodules in each plant species also decreased with plant age due to application of NPK fertilizer. The maximum dry matter of nodules of the legumes were observed at the onset of flowering stage and decreased sharply over

time. Dry weight of nodules of long bean grown without fertilizer was higher (0.07 g/plant) at flower initiation stage whilst long bean grown with fertilizer recorded lower dry weight of nodules (0.02 g/plant). Mung bean grown without fertilizer produced higher nodule dry matter (0.01 g/plant) at flower initiation stage than with fertilizer (Figure 4.8) (Appendix 2).

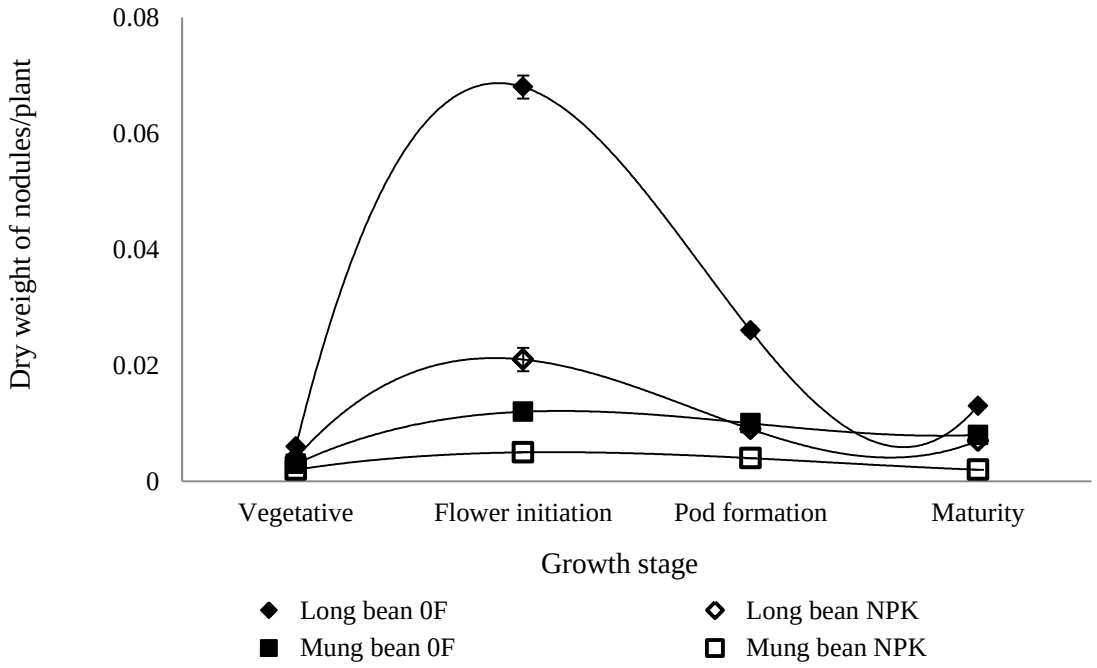

Figure 4.8. Nodule dry weight of Long bean and Mung bean at different growth stages.
Vertical bars represent LSD at $p<0.05$ among the treatments

4.5 Grain yield and yield attributes

Both long bean and mung bean grown without fertilizer produced significantly higher pods per plant than the beans grown with fertilizer. Seeds per pod, 100-seed weight as well as seeds yield were identical between long bean and mung bean (Table 4.2).

Table 4.2. Pods per plant, seeds per pod, 100-seed weight and grain yield of Long bean and Mung bean at maturity stage.

Treatment	Pods per plant (no.)	Seeds per pod (g plant^{-1})	100-seed weight (g)	Grain yield (g plant^{-1})
Long bean				
Control	$5.83^{a} \pm 0.27$	$13.75^{ns} \pm 0.52$	$12.68^{ns} \pm 0.22$	$10.07^{ns} \pm 0.76$
Fertilizer	$5.50^{b} \pm 0.22$	$13.72^{ns} \pm 0.44$	$10.07^{ns} \pm 0.19$	$10.21^{ns} \pm 0.45$
Mung bean				
Control	$5.80^{a} \pm 0.43$	$11.55^{ns} \pm 0.45$	$4.80^{ns} \pm 0.09$	$3.62^{ns} \pm 0.09$
Fertilizer	$5.77^{b} \pm 0.17$	$11.57^{ns} \pm 0.49$	$4.78^{ns} \pm 0.06$	$3.65^{ns} \pm 0.11$

Means followed by the same letters are not significantly different for each treatment means ($P < 0.05$) by LSD, ns = non-significant

4.6 Total dry matter accumulation

Long bean and mung bean grown without fertilizer produced slightly higher total dry matter than crops grown with fertilizer (Table 4.3).

Table 4.3. Total dry matter per plant of long bean and mung bean

Treatment	Dry matter (g plant $^{-1}$) Maturity stage
Long bean	
Control	$32.93^{ns} \pm 0.24$
Fertilizer	$31.54^{ns} \pm 0.44$
Mung bean	
Control	$21.78^{ns} \pm 0.15$
Fertilizer	$20.30^{ns} \pm 0.12$

Means followed by the same letters are not significantly different for each treatment means ($P < 0.05$) by LSD, ns = non-significant

4.7 Nitrate reductase activity

Nitrate reductase activity was affected by the treatment variables and it was decreased with the increase of plant age. Nitrate reductase activity was higher at the initiation of flowering stage. The highest NRA was found in nodules of long bean grown without fertilizer (17.65 µmols NO_2^- h^{-1} $gfwt^{-1}$ at 40 DAE) and the lower NRA (6.68 µmols NO_2^- h^{-1} $gfwt^{-1}$) was recorded by nodules of mung bean grown with fertilizer. Long bean grown with fertilizer and mung bean grown without fertilizer obtained 7.73 µmols NO_2^- h^{-1} $gfwt^{-1}$ at 36 DAE and 12.03 µmols NO_2^- h^{-1} $gfwt^{-1}$ at 42 DAE, respectively (Figure 4.9).

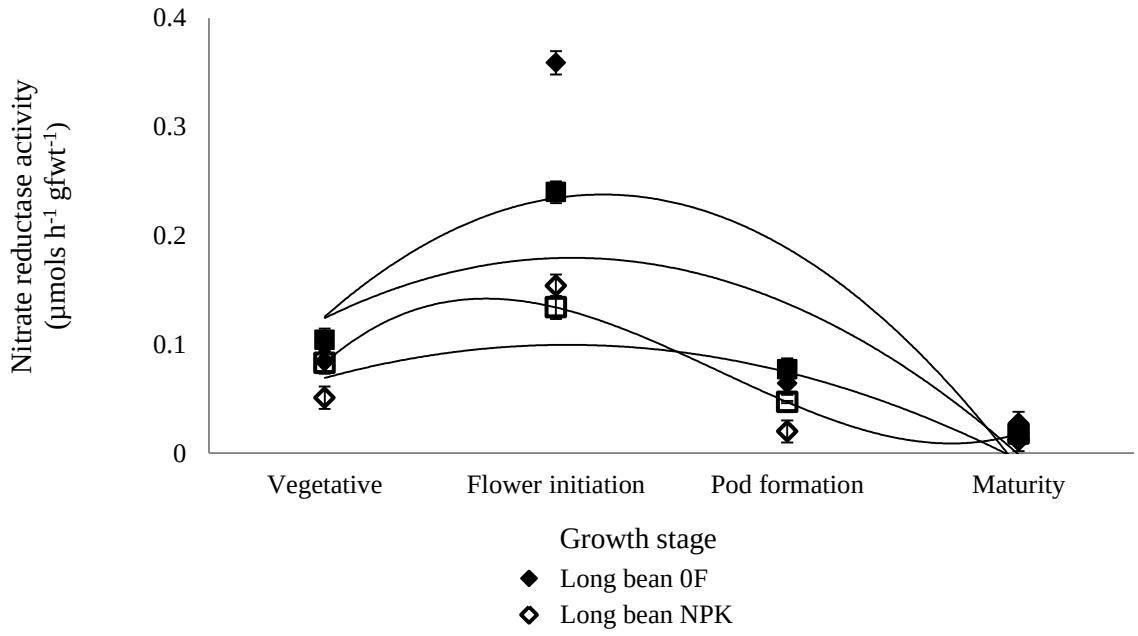

Figure 4.9. The nitrate reductase activity in nodules of Long bean and Mung bean at different growth stages
Vertical bars represent t-test at $p<0.05$ among the treatments

CHAPTER 5

DISCUSSION

Fertilizer application gave positive effects to chlorophyll content and photosynthetic activity of crops grown with fertilizer whilst negative effects on nodule production and NRA in nodules. Soil is a massive pool of bacteria (Achakzai, 2007), which is prominent in biological nitrogen fixation (BNF). There was a little difference of N content in soil of the legumes grown with and without fertilizer. This showed that NPK fertilizer did not take effect on both long bean and mung bean. Nitrogen, K and P are primary nutrients which are deficient in soil due to uptake by plants for growth and survival. Calcium, Mg as well as S are usually sufficient in the soil. They are often naturally maintained in the soil in sufficient quantities where supplementation with fertilizers is unnecessary (Robertson, 2013).

Both legumes possessed the highest chlorophyll content at the initiation of flowering stage and declined thereafter. At later stages, the chlorophyll content decreased due to translocation of carbohydrate from leaves into the nodules that provides N for plant growth. The carbohydrate is used to feed the *Rhizobium* bacteria (Biederbeck *et al.*, 2005). Crops grown with fertilizer obtained higher chlorophyll content than crops grown without fertilizer as a result of NPK fertilizer application. Many researchers proved that there is a very close link between N and chlorophyll content (Daughtry, 2000; Tucker, 2004; Amaliotis *et al.*, 2004). The chlorophyll synthesis depends on mineral nutrition (Daughtry, 2000) where N is responsible for the leaf growth and one of the constituent elements of chlorophyll molecule that contains 4 N in tetrapyrolle ring. Nitrogen also acts as a prominent element in green leaves and related to

chlorophyll content (Haboudane *et al.*, 2002) and in protein molecules which affects the chloroplast formation (Daughtry, 2000). Evans (1983) revealed that the chlorophyll content is approximately proportional to N content in the leaf. Phosphorus affects the chlorophyll molecules stability in plants and K plays role indirectly in the chlorophyll synthesis by enhancing the uptake of N, Fe^{2+}, Mg^{2+} and SO_4^{2-} (Gairola *et al.,* 2009). Long bean grown with fertilizer obtained the highest chlorophyll content due to the highest chlorophyll *a* content. Mung bean grown without fertilizer produced poor total chlorophyll content due to lowest chlorophyll *a* content. This is possibly due to chlorophyll *a* content able to capture a limited wavelength only. Mung bean grown without fertilizer produces more chlorophyll *b* which leads to the highest chlorophyll *a/b* ratio content in order to increase its photosynthetic ability because chlorophyll *b* can capture a wider range of light. Our results coincided with the findings of Kumar (2009) who recorded maximum chlorophyll *a*/*b* ratio in control plants.

At onset of flowering, both legume crops recorded the highest photosynthetic rate and then decreased with increase of plant age. At this stage, higher stomatal conductance in leaves gives rise to diffusion of CO_2 which favors higher photosynthetic rates (Flexas *et al.,* 2002). The photosynthetic rate decreases at later growth stages due to chloroplast lose its integrity, lower stomatal conductance and higher intercellular CO_2 concentration (Biswas *et al.,* 2001). Decrease in chlorophyll *a* content leads to decrease in photosynthetic activity. This is because chlorophyll *a* molecules are vital in photosynthesis energy phase that are necessary before photosynthesis begin. Long bean and mung bean grown with fertilizer obtained higher photosynthetic activities compared to long bean and mung bean grown without fertilizer. Bojović and Marković

(2007) also observed that the lowest leaf chlorophyll content obtained in plants grown without fertilizer in soil. This is because NPK fertilizer takes effect on crops grown with fertilizer in photosynthesis process. It increases the leaf area of plants (Evans, 1989) and helps in uptake of NO_3 and K ions which leads to increase in chlorophyll content. Nitrogen, P and K are involved in photosynthesis process (Haboudane *et al.*, 2002). Nitrogen within leaf tissue is a major determinant of photosynthesis where N inputs stimulates the N_2 fixation and facilitates the photosynthetic enhancements (Peterson *et al.*, 1999). Nitrogen is required to build amino acids for proteins. Proteins are required for cell growth, enzymes and cell membranes. Phosphorus is involved in respiration and photosynthesis. It influences the photosynthesis activity of crops by modulating directly or indirectly the activated RUBISCO content (Kumar, 2009). This element is also required to make cell membranes and DNA. Potassium is required by enzymes which involved in respiration and photosynthesis. The highest photosynthetic activity was found in long bean grown with fertilizer due to the highest total chlorophyll and chlorophyll *a* content. Hesketh *et al.*, (1981) demonstrated a positive correlation between leaf photosynthetic rate and chlorophyll content.

Crops grown without fertilizer possessed higher total dry matter than crops grown with fertilizer. Higher crop yield and biomass were obtained as a result of higher net CO_2 assimilation rates (Flexas *et al.*, 2002). Total dry weights of mung bean grown with and without fertilizer were almost identical which means that NPK fertilizer did not response well on mung bean. NPK fertilizer influences crop growth and dry matter production. Nitrogen affected the dry matter accumulation (Hu *et al.*, 2010) in legumes and gave positive effect to accumulation of N in grain which was derived by

translocation of N in vegetative organs during pod filling period (Cartelle *et al.*, 2006). Nitrogen is responsible for leaf growth and increased N in dry matter of plants (Reynolds, 2005). Phosphorus is responsible for root development while K is responsible for flower and fruit development and increases the dry weight of plants (Arrese-Igor *et al*., 1998). Sharma *et al.*, (2000) stated that promoting effect of NPK fertilizer application attributed to increase of the dry matter compared to control which reflected in the increase in the yield and yield components (Agamy *et al.*, 2012).

In this study, crops grown without fertilizer successfully obtained higher number of pods and seed yield compared to crops grown with fertilizer. Mung bean grown without fertilizer recorded the highest number of pods whilst long bean grown without fertilizer obtained the highest seed yield. Long bean grown without fertilizer obtained the highest dry weight of pods due to translocation of carbohydrates from nodules into the seed production which involved in the formation of pods. These nodules then contributed to the pods production and led to the highest pods dry matter. It is reported that large nodules contribute a greater sink for pods production (Franzen, 1999). On the other hand, mung bean grown with fertilizer obtained slightly higher dry weight of pods than mung bean grown without fertilizer. NPK fertilizer application affected the pods growth of mung bean grown with fertilizer where P is vital in fruit and pod development.

Number of nodules and nodule dry weight were the highest at the onset of flowering stage in long bean and mung bean grown with and without fertilizer and declined at later stages due to “source-sink relationship” where carbohydrate translocated from nodules sink to pod formation. Crops grown with fertilizer obtained less number of

nodules and dry weight of nodules than crops grown without fertilizer as a consequence of application of NPK fertilizer (Appendix 4). This could be the fact that N may depress or inhibits the nodules production. The size of nodules in crops grown without fertilizer was bigger than the fertilized crops. Javaid (2009) reported that crops grown in un-amended soil recorded the highest number of nodules. Previous studies proved that application of higher rates of N fertilizer may results in decrease in dry and fresh nodules weight (Olson *et al.,* 1981; Eriksen and Whitney, 1984). Poor number of nodules produced when N fertilizer applied compared to control treatment (Carr, 2000). The higher application rates of N fertilizer resulted in linear decrease of nodules dry weight as a consequence of regulatory mechanism (Reynolds, 2005; Achakzai, 2007). Varges *et al.,* (2000) stated that application of N fertilizer decreased the nodules number and had no effect on early formation of nodules. Plant reduces number of nodules through a regulatory mechanism which favors older nodules while the younger nodules suppress (Stougaard, 2000). Previous studies revealed that fertilizer application is not advocated in maximizing the N_2 fixation in the soil (Varges *et al.,* 2000; Reynolds, 2005; Achakzai, 2007). Inhibition of nitrogenase and NR activities in nodules of legume plants by NO_3 has been well known. The negative effect of NO_3 on root nodulation can be reflected through the ratio of dry mass of nodules to the whole mass of plant (Streeter, 1988). The concentration of free oxygen within the infected zone of root nodules is the major factor that affects nitrogenase activity (Layzell and Hunt, 1990).

The activity of nitrate reductase and haemoglobin content in nodules are reduced when the age of nodules approached senescence stage (Caba *et al*., 1990). The NRA in

nodules of long bean and mung bean grown without fertilizer were found higher than long bean and mung bean grown with fertilizer. The highest peak was obtained by long bean grown without fertilizer at the onset of flowering. This result is reflected the production of the highest number of nodules and dry matter than produced by long bean grown with fertilizer. Nitrate is the main form of N that absorbed from the soil by plants which then translocated from root to shoot (Olday *et al.,* 1976; Gairola *et al.,* 2009). Nitrate reductase activity positively correlated with leaf NO_3 content and regulated by various factors such as plant growth regulators, light, temperature, nitrite (NO_2), and carbon dioxide (CO_2) (Luo *et al.,* 1991). Generally, NRA is higher in the nodules than in other plant parts (Ashraf and Iram, 2005). The increment in NRA is due to de novo synthesis of mRNA and enzyme in addition to post translational regulation by dephosphorylation and phosphorylation. The highest NRA was obtained from the nodules at the initiation of flowering and declined thereafter. Nitrate is reduced to NO_2 by NR. The NO_3 accumulation and assimilation in cell depends on NR activity. The NRA was the greatest when legumes were grown in the absence of inorganic N. Nitrate reductase activity in nodules is related to increment of N content by legumes and the haemoglobin content of nodules (Caba *et al*., 1990) where root nodules are able to reduce NO_3 accumulation rapidly (Giannakis *et al.,* 1988). Gairola *et al.,* (2009) found that an increase in NRA decreases the accumulation of nitrate. Without inorganic fertilizer application, crops able to obtain high NRA that contribute in production of nodules which helps in maintaining soil fecundity via N_2 fixation process for the next crop. A reverse relationship is observed between NRA and NO_3 content (Olday *et al.,* 1976). It shows that application of inorganic N fertilizer such as

urea or NH_4 may increase NO_3 accumulation in plants that lead to suppression of N_2 fixation (Benavides *et al.*, 2011).

Achakzai (2007) stated that the number of nodules and nitrate reductase activities decreases when the selected crop is treated with N fertilizers. This is because of the fact that formation of nodules is inhibited with the application of N which leads to limitation of N_2 fixation where roots do not allow bacterial infection. A byproduct of NRA, NO_2, hinders the function of leghaemoglobin as well as nitrogenase (Becana and Sprent, 1987). This can be observed due to accumulation of toxic levels of NO_2 from the nitrate reductase reaction (Caba *et al*., 1990). It was strongly suggested that NO_3 accumulation affects the N_2 fixation process through formation of NO_2 and binding of leghaemogloebin (Lb) to form nitrosyl-Lb, which is unable to bind O_2 (Arrese-Igor *et al*., 1998). Inorganic N fertilizer (NO_3 or NH_4) treatment resulted in decrease of nodules nitrate reductase activity as well as hemoglobin content (Caba *et al*., 1990). Application of N fertilizer that has been practiced by Asian farmers is reported to inhibit N_2 fixation where urea inhibited acetylene reduction assay (ARA) and rates of N fixation (Gaulke *et al.*, 2006).

CHAPTER 6

CONCLUSIONS

Long bean grown with fertilizer recorded the highest total chlorophyll (2.99 mg/g), leaf SPAD value (41.35), chlorophyll *a* content (2.25 mg/g), photosynthetic activity (1.26 µmol m^{-2} s^{-1}) and number of pods whilst long bean grown without fertilizer obtained the highest number of nodules and nodule dry weight, pod dry weight and NRA in nodules (17.65 µmols NO_2^- h^{-1} $gfwt^{-1}$).

Crops grown without fertilizer possessed better root nodulation based on NRA in nodules whilst crops grown with fertilizer obtained weak root nodulation. This is because N fertilizer application interrupts the N_2 fixation process due to NO_3 accumulation within the plants which inhibits the conversion of N_2 to NH_3. The results revealed that long bean has higher chlorophyll content and photosynthetic activity than mung bean. Long bean is also a better N accumulator due to better root system where lots of soil microbes can be attracted. Long bean and mung bean grown without fertilizer performed better nodulation which provided addition of N into soil compared to crops grown with fertilizer.

Macronutrients can be maintained naturally in sufficient quantities which are required for healthy growth of crops. Application of NPK fertilizer is not necessary to be used to grow long bean and mung bean on fertile soil. The usage of highly cost inorganic N fertilizer might causes environmental and health problems and it needs to be halt.

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SUPPLEMENTARY

LIST OF PUBLICATION

Nursu'aidah, H., Motior, M. R., Nazia, A. M. and Islam, A. M. (2014). Growth and photosynthetic responses of long bean (*Vigna unguiculata*) and mung bean (*Vigna radiata*) response to fertilization. *The Journal of Animal and Plant Sciences, 24*(2), 573 – 578.

APPENDIX 1

SOLUTIONS:

i) 80% Acetone

Mix 80 mL of acetone with 20 mL of distilled water (Total volume = 100 mL).

Assay medium for NRA

ii) NO_2 stock solution (250 µm standard solution)

Dissolve 0.173 g of sodium nitrite ($NaNO_2$, 99.99%) in 100 mL distilled water. Prepare in a 100 mL volumetric flask.

iii) 0.01M phosphate buffer (pH 7.0) containing 0.02M KNO_3

A total of 3.32 g of K_2HPO_4 dissolved in 300 mL distilled water in a beaker. The pH adjusted by adding KH_2PO_4 until the buffer pH reached pH 7. Place the buffer solution into 500mL volumetric flask and top up with distilled water until the level of water reached the 500 mL mark. Add 1.011 g of potassium nitrate into the buffer solution and stir with magnetic stirrer.

iv) 1 % sulfanilamide solution (SA)

Dissolve 5 g of sulfanilamide (powder) in 100 mL distilled water. Mix with 50 mL of concentrated HCl and top pup the SA solution until it reached 500 mL. This solution is stable for long period.

v) 0.02% N-1-Naphthylethylenediamine dihydrochloride solution (NEED)

Dissolve 0.10 g of NEED in 500 ml of distilled water.

APPENDIX 2

Table 4.8: ANOVA of chlorophyll contents of long bean and mung bean over time

Total chlorophyll

Long bean **Mung bean**

Vegetative

SV	DF	SS	MS
Treatment	1	0.13	0.13^{ns}
Error	10	4.3	0.43
Total	11	4.43	

SV	DF	SS	MS
Treatment	1	0.15	0.15^{ns}
Error	10	10.07	1.01
Total	11	10.22	

Flower initiation

SV	DF	SS	MS
Treatment	1	0.01	0.01^{ns}
Error	10	1.67	0.17
Total	11	1.68	

SV	DF	SS	MS
Treatment	1	0.57	0.57^{ns}
Error	10	4.49	0.45
Total	11	5.06	

Pod formation

SV	DF	SS	MS
Treatment	1	0.46	0.46^{ns}
Error	10	1.86	0.19
Total	11	2.32	

SV	DF	SS	MS
Treatment	1	0.19	0.19^{ns}
Error	10	2.24	0.22
Total	11	2.43	

Maturity

SV	DF	SS	MS
Treatment	1	0.36	0.36^{ns}
Error	10	2.45	0.25
Total	11	2.81	

SV	DF	SS	MS
Treatment	1	0.9	0.9^{ns}
Error	10	2.98	0.3
Total	11	3.88	

* - significance level at 5% ** - significance level at 1% ns – non-significant

Chlorophyll *a*

Long bean **Mung bean**

Vegetative

SV	DF	SS	MS
Treatment	1	0.07	0.07^{ns}
Error	10	0.3	0.03
Total	11	0.37	

SV	DF	SS	MS
Treatment	1	0.002	0.002^{ns}
Error	10	0.73	0.07
Total	11	0.728	

Flower initiation

SV	DF	SS	MS
Treatment	1	0.01	0.01^{ns}
Error	10	0.92	0.09
Total	11	0.91	

SV	DF	SS	MS
Treatment	1	0.62	0.62^{ns}
Error	10	2.22	0.22
Total	11	2.84	

Pod formation

SV	DF	SS	MS
Treatment	1	0.18	0.18^{ns}
Error	10	1.02	0.1
Total	11	1.2	

SV	DF	SS	MS
Treatment	1	0.09	0.09^{ns}
Error	10	1.26	0.13
Total	11	1.35	

Maturity

SV	DF	SS	MS
Treatment	1	0.15	0.15^{ns}
Error	10	1.38	0.14
Total	11	1.53	

SV	DF	SS	MS
Treatment	1	0.5	0.5^{ns}
Error	10	1.58	0.16
Total	11	2.08	

* - significance level at 5% ** - significance level at 1% ns – non-significant

Chlorophyll *b*

Long bean **Mung bean**

Vegetative

SV	DF	SS	MS
Treatment	1	0.01	0.01^{ns}
Error	10	0.06	0.006
Total	11	0.07	

SV	DF	SS	MS
Treatment	1	0.004	0.004^{ns}
Error	10	0.076	0.008
Total	11	0.08	

Flower initiation

SV	DF	SS	MS
Treatment	1	0.001	0.001^{ns}
Error	10	0.128	0.013
Total	11	0.129	

SV	DF	SS	MS
Treatment	1	0.001	0.001^{ns}
Error	10	0.439	0.04
Total	11	0.44	

Pod formation

SV	DF	SS	MS
Treatment	1	0.072	0.072*
Error	10	0.13	0.013
Total	11	0.202	

SV	DF	SS	MS
Treatment	1	0.02	0.02^{ns}
Error	10	0.15	0.02
Total	11	0.17	

Maturity

SV	DF	SS	MS
Treatment	1	0.05	0.05^{ns}
Error	10	0.17	0.017
Total	11	0.22	

SV	DF	SS	MS
Treatment	1	0.06	0.06^{ns}
Error	10	0.23	0.02
Total	11	0.29	

* - significance level at 5% ** - significance level at 1% ns – non-significant

Chlorophyll *a/b* ratio

Long bean **Mung bean**

Vegetative

SV	DF	SS	MS
Treatment	1	0.001	0.001^{ns}
Error	10	0.006	0.0006
Total	11	0.007	

SV	DF	SS	MS
Treatment	1	0.001	0.001^{ns}
Error	10	0.009	0.0009
Total	11	0.01	

Flower initiation

SV	DF	SS	MS
Treatment	1	0.004	0.004^{ns}
Error	10	0.016	0.002
Total	11	0.02	

SV	DF	SS	MS
Treatment	1	0.03	0.03*
Error	10	0.03	0.003
Total	11	0.06	

Pod formation

SV	DF	SS	MS
Treatment	1	0.014	0.014*
Error	10	0.022	0.002
Total	11	0.036	

SV	DF	SS	MS
Treatment	1	0.01	0.01^{ns}
Error	10	0.09	0.009
Total	11	0.1	

Maturity

SV	DF	SS	MS
Treatment	1	0.013	0.013*
Error	10	0.013	0.0013
Total	11	0.026	

SV	DF	SS	MS
Treatment	1	0.002	0.002^{ns}
Error	10	0.028	0.003
Total	11	0.03	

* - significance level at 5% ** - significance level at 1% ns – non-significant

Chlorophyll content (SPAD value)

Long bean **Mung bean**

Vegetative

SV	DF	SS	MS
Treatment	1	28.21	28.21^{ns}
Error	10	76.76	7.676
Total	11	104.97	

SV	DF	SS	MS
Treatment	1	15.64	15.64^{ns}
Error	10	70.31	7.03
Total	11	85.95	

Flower initiation

SV	DF	SS	MS
Treatment	1	25.81	25.81^{ns}
Error	10	167.87	16.79
Total	11	193.68	

SV	DF	SS	MS
Treatment	1	18.5	18.5^{ns}
Error	10	102.25	10.23
Total	11	120.75	

Pod formation

SV	DF	SS	MS
Treatment	1	58.97	58.97*
Error	10	92.5	9.25
Total	11	151.47	

SV	DF	SS	MS
Treatment	1	12.2	12.2^{ns}
Error	10	78.87	7.89
Total	11	91.07	

Maturity

SV	DF	SS	MS
Treatment	1	44.47	44.47*
Error	10	212.05	21.21
Total	11	256.52	

SV	DF	SS	MS
Treatment	1	17.04	17.04^{ns}
Error	10	96.58	9.66
Total	11	113.62	

* - significance level at 5% ** - significance level at 1% ns – non-significant

Photosynthetic rate

Long bean

Vegetative

SV	DF	SS	MS
Treatment	1	0.14	0.14**
Error	10	0.08	0.008
Total	11	0.22	

Flower initiation

SV	DF	SS	MS
Treatment	1	0.36	0.36ns
Error	10	1.25	0.13
Total	11	1.61	

Pod formation

SV	DF	SS	MS
Treatment	1	0.22	0.22*
Error	10	0.27	0.03
Total	11	0.49	

Maturity

SV	DF	SS	MS
Treatment	1	0.04	0.04ns
Error	10	0.11	0.01
Total	11	0.15	

Mung bean

Vegetative

SV	DF	SS	MS
Treatment	1	0.02	0.02*
Error	10	0.02	0.002
Total	11	0.04	

Flower initiation

SV	DF	SS	MS
Treatment	1	0.06	0.06ns
Error	10	1.08	0.11
Total	11	10.72	

Pod formation

SV	DF	SS	MS
Treatment	1	0.001	0.001ns
Error	10	0.069	0.0069
Total	11	0.07	

Maturity

SV	DF	SS	MS
Treatment	1	0.005	0.005ns
Error	10	0.112	0.011
Total	11	0.117	

* - significance level at 5% ** - significance level at 1% ns – non-significant

Dry weight of nodules

Long bean **Mung bean**

Vegetative

SV	DF	SS	MS
Treatment	1	0.00001	0.00001^{ns}
Error	10	0.00003	0.000003
Total	11	0.00004	

SV	DF	SS	MS
Treatment	1	0.00001	0.00001*
Error	10	0.00001	0.000001
Total	11	0.00002	

Flower initiation

SV	DF	SS	MS
Treatment	1	0.007	0.007^{ns}
Error	10	0.036	0.004
Total	11	0.043	

SV	DF	SS	MS
Treatment	1	0.00008	0.00008^{ns}
Error	10	0.00042	0.00004
Total	11	0.0005	

Pod formation

SV	DF	SS	MS
Treatment	1	0.0008	0.0008^{ns}
Error	10	0.016	0.002
Total	11	0.017	

SV	DF	SS	MS
Treatment	1	0.00011	0.00011*
Error	10	0.00015	0.000015
Total	11	0.00026	

Maturity

SV	DF	SS	MS
Treatment	1	0.0001	0.0001^{ns}
Error	10	0.0003	0.00003
Total	11	0.0004	

SV	DF	SS	MS
Treatment	1	0.0001	0.0001^{ns}
Error	10	0.001	0.0001
Total	11	0.0011	

* - significance level at 5% ** - significance level at 1% ns – non-significant

APPENDIX 3

2.1.2.1 Long bean

Flower

Pod

2.1.2.2 Mung bean

Flowers

Pods

CHAPTER 3

3.1 Experimental site and management

Seeds (from MARDI)

Urea (N)

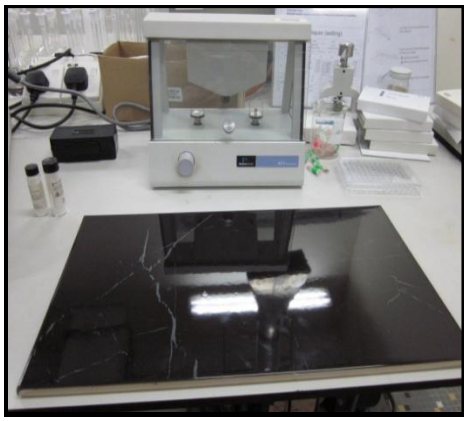
Triple superphosphate (P)

Muriate of Potash (K)

3.2.1 CHN analysis

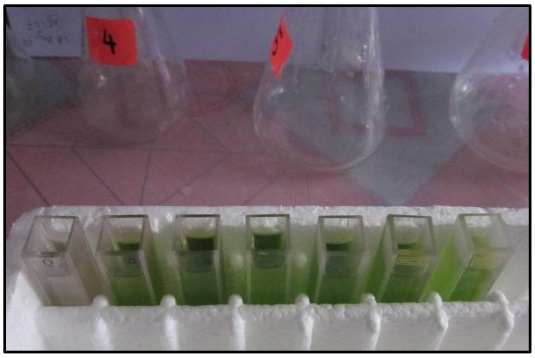
Tin capsules

CHN analyzer



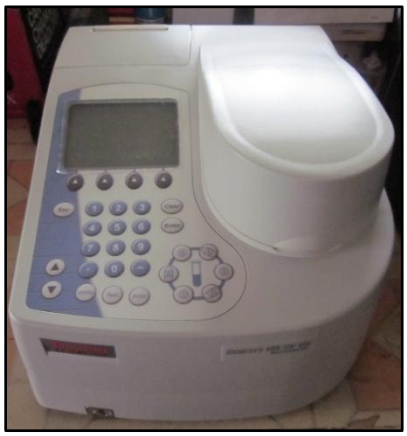
Weighing balance

3.3 Chlorophyll content determination



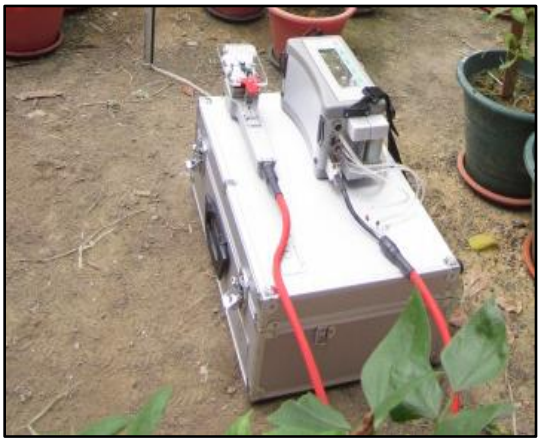
Arnon's method

SPAD meter



Spectrophotometer

3.4 Photosynthetic rate



Lci – SD

3.5 Dry matter yield

Oven

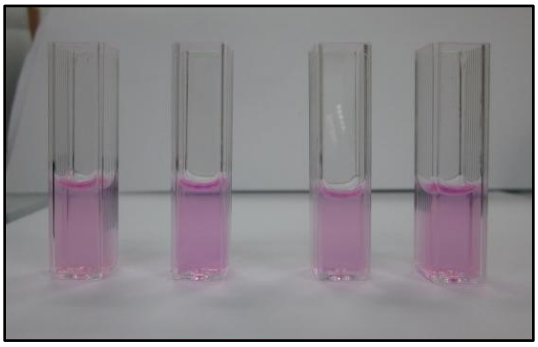
Weighing balance

3.6 Preservation of nodules

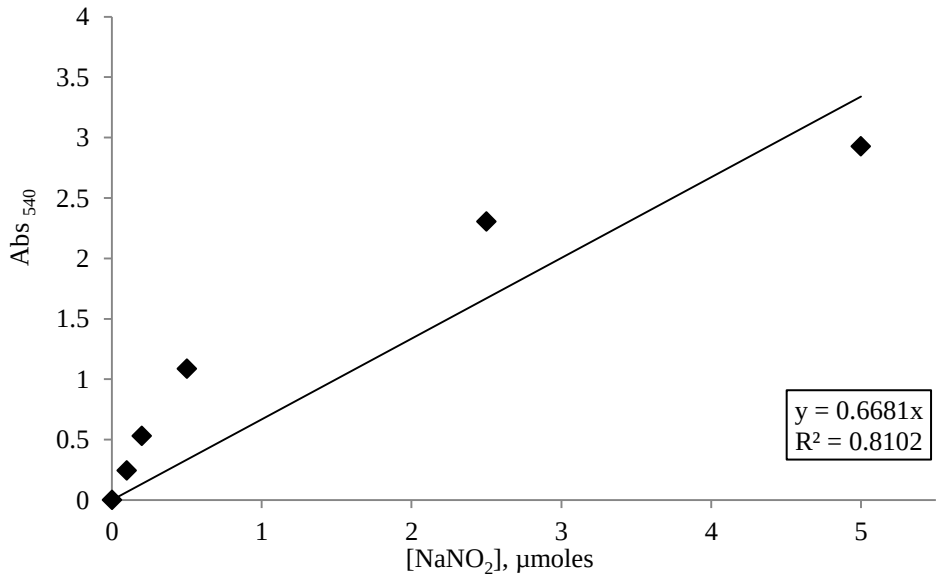
Nodules (in vials)

3.7 Determination of nitrate reductase activity (NRA)

No fertilizer (0F)



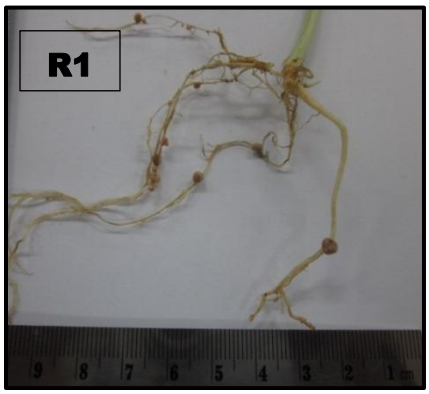
Treated NPK

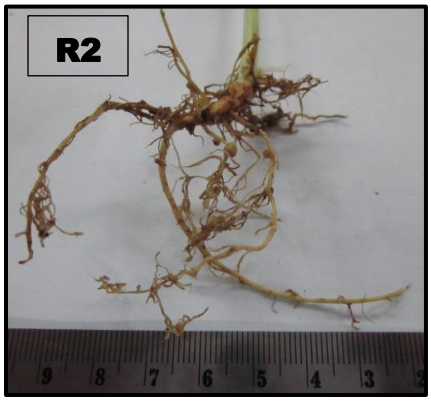


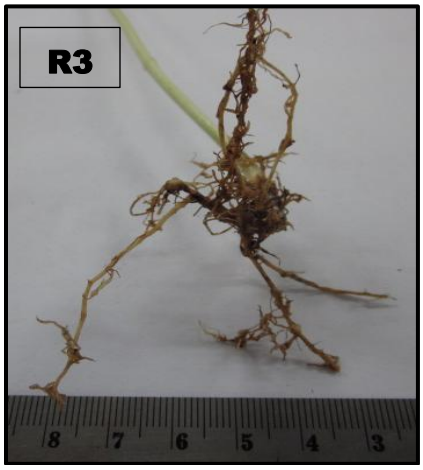
NO_2 standard curve (A_{540} versus µmoles NO_2)

APPENDIX 4

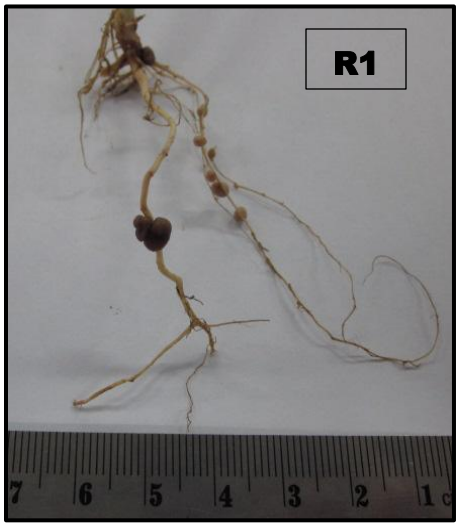
A) Long bean [No fertilizer applied (0F)]
i) Before flowering

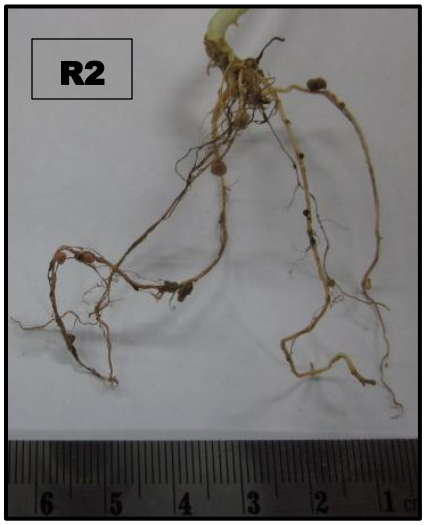


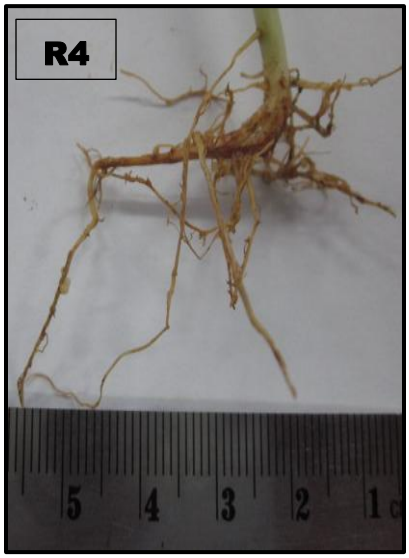




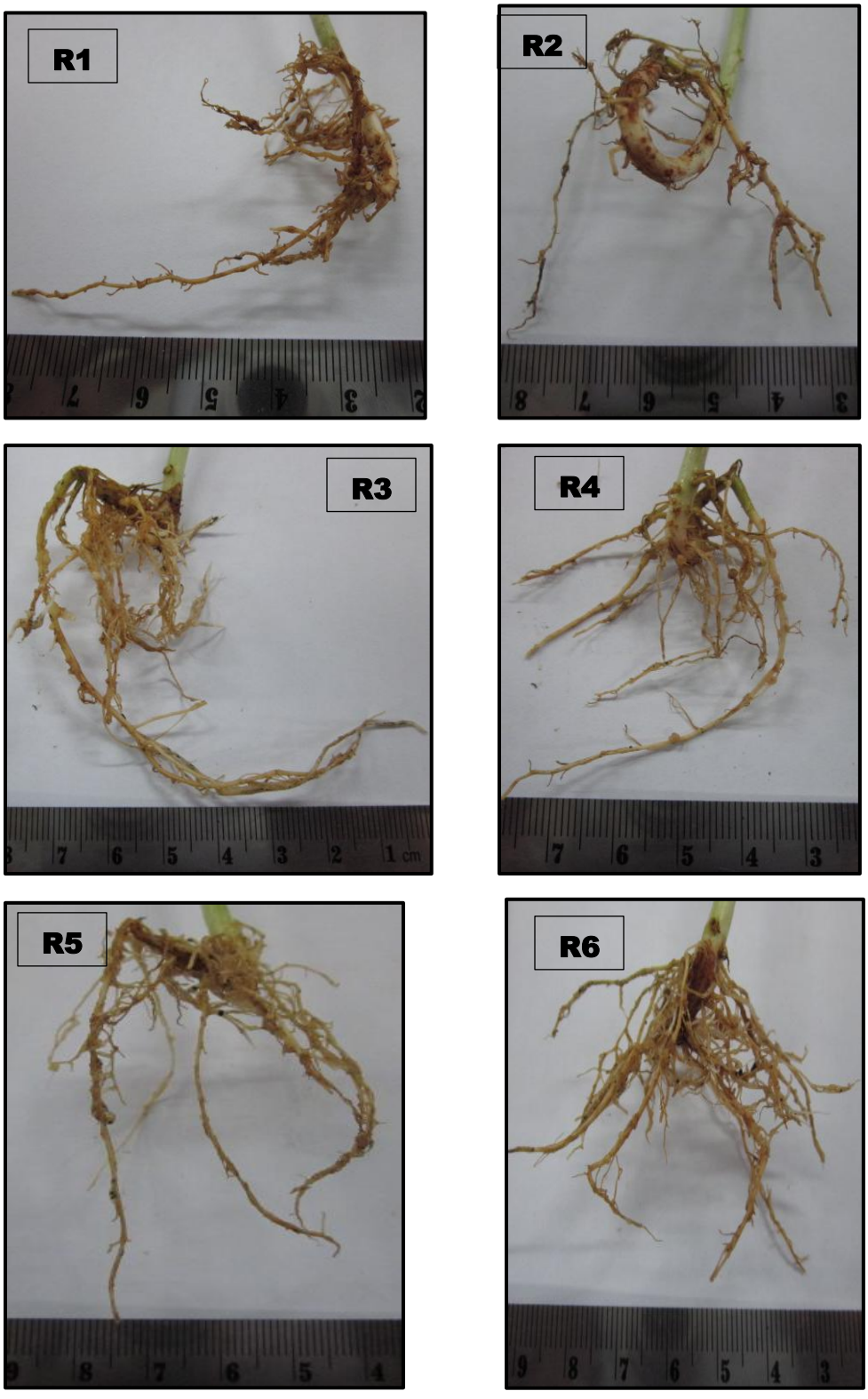
ii) Initiation of flowering



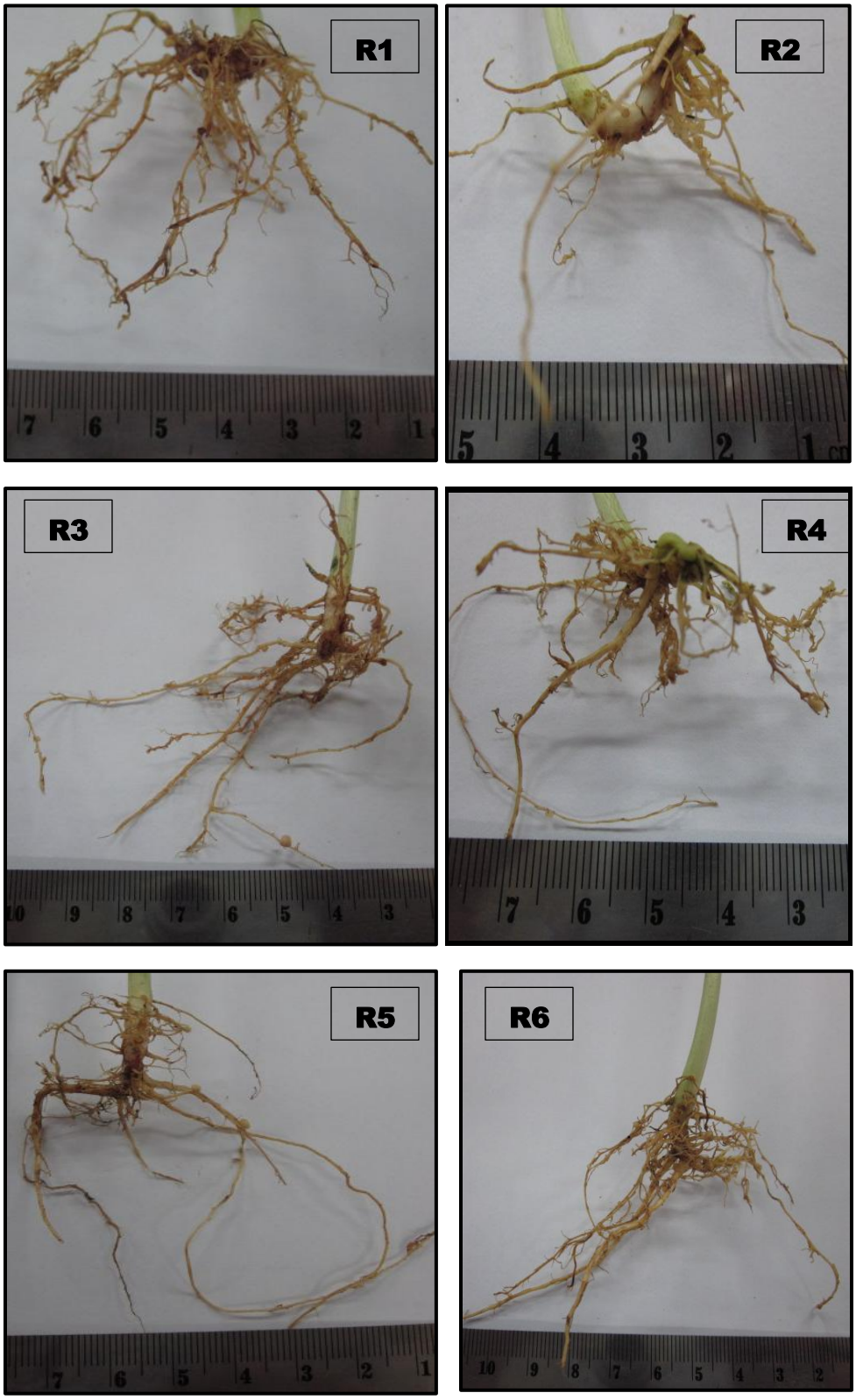




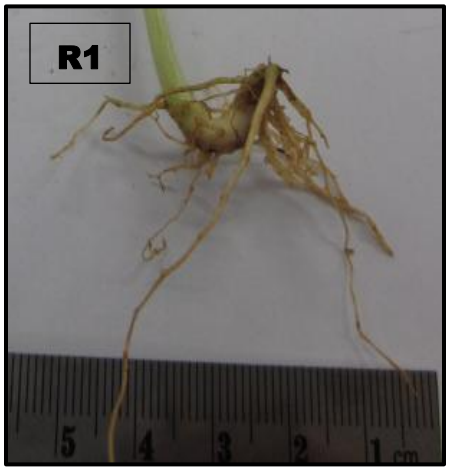
iii) Pod formation

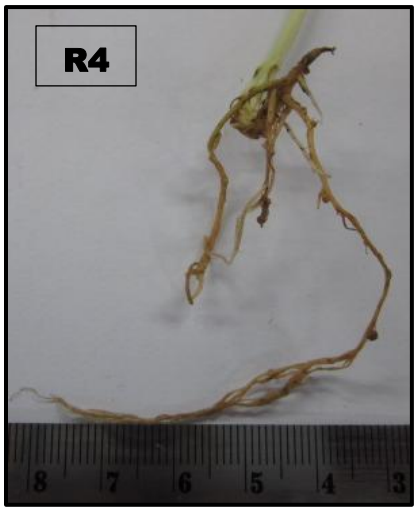


iv) Maturity



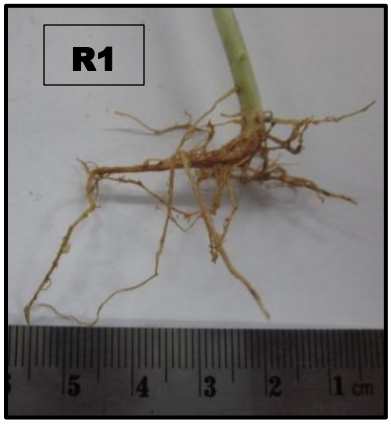
B) Long bean [Treated NPK (NPK)]
i) Before flowering

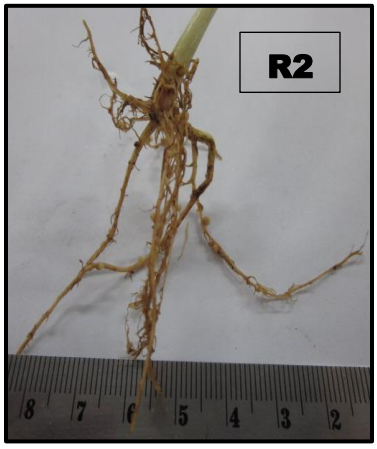




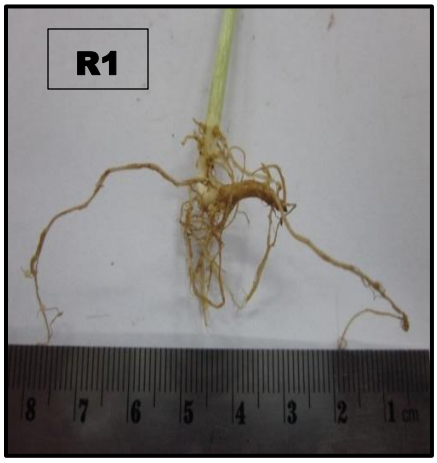
ii) Initiation of flowering

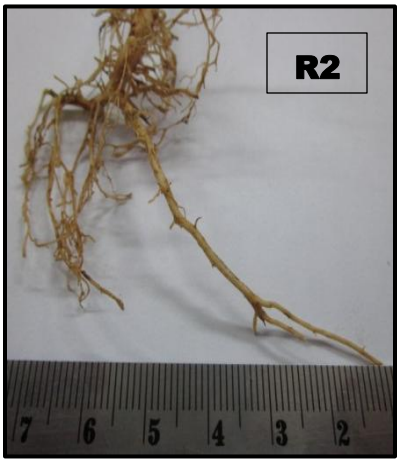
iii) Pod formation



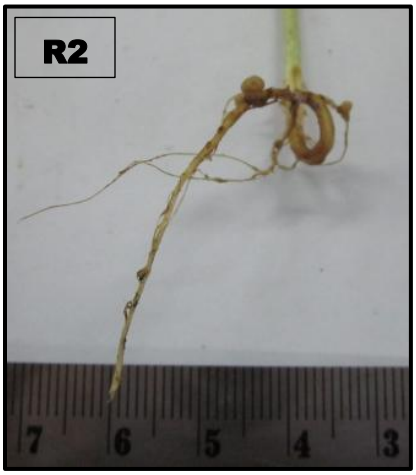


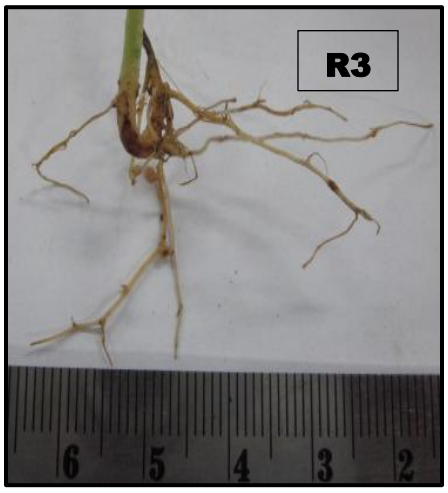
iv) Maturity

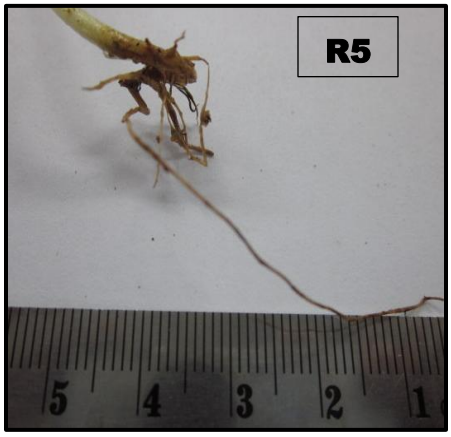


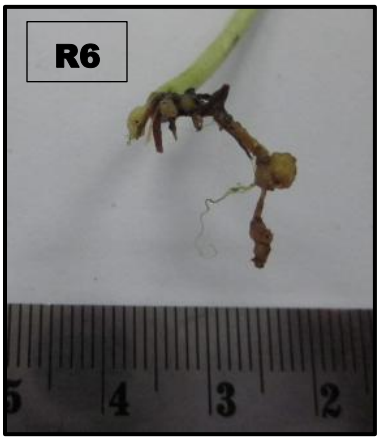


C) Mung bean [No fertilizer applied (0F)]
i) Before flowering

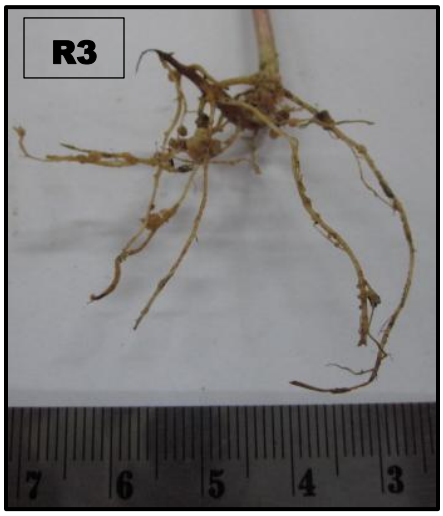


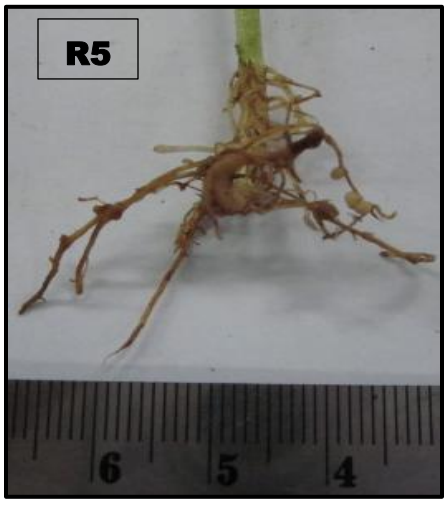


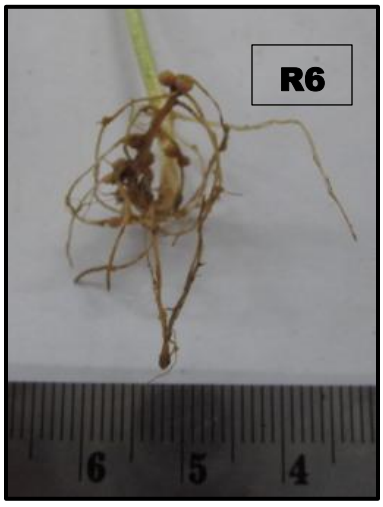




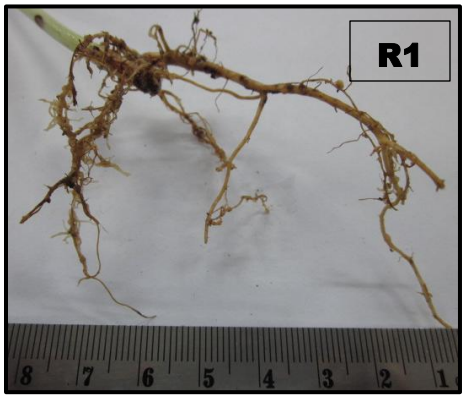
ii) Initiation of flowering

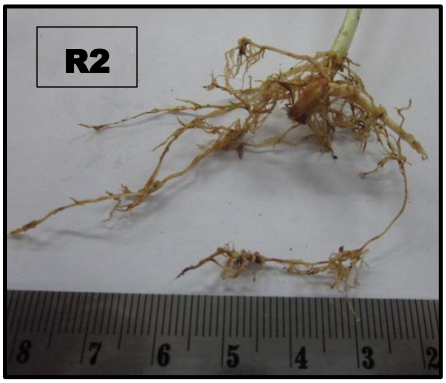


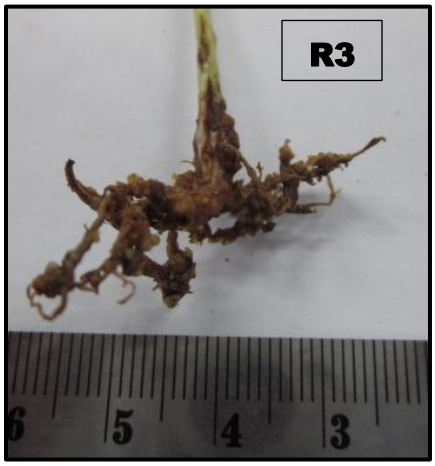


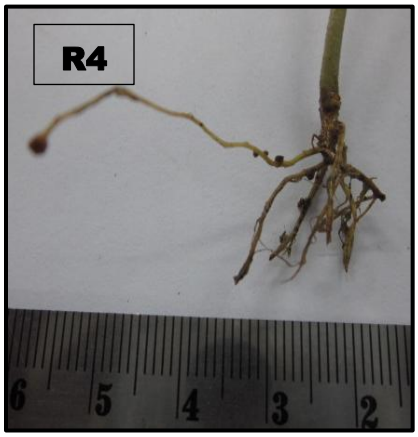


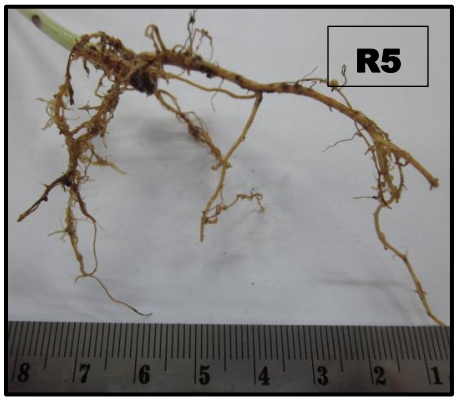
iii) Pod formation



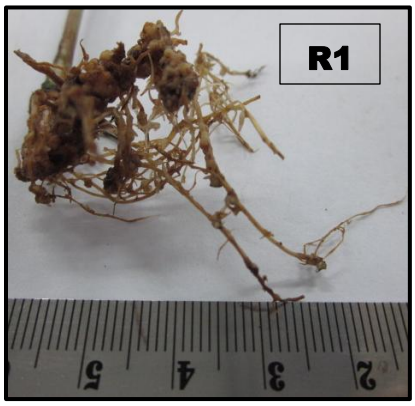


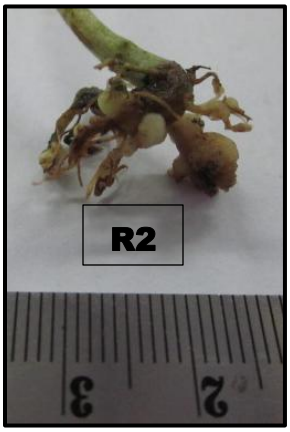


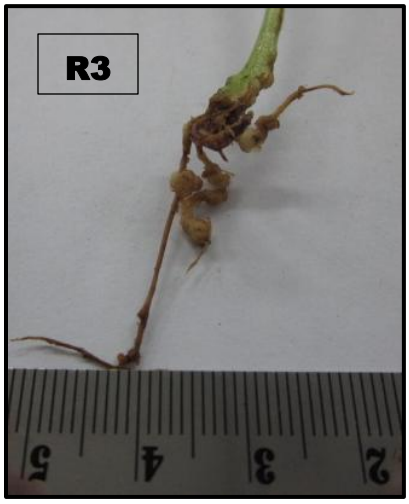


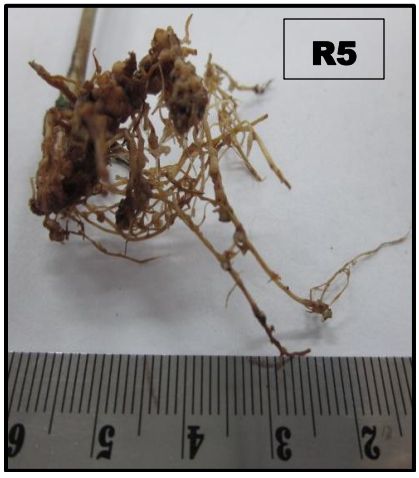


iv) Maturity



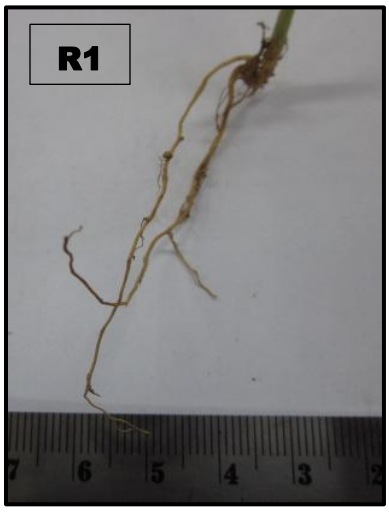


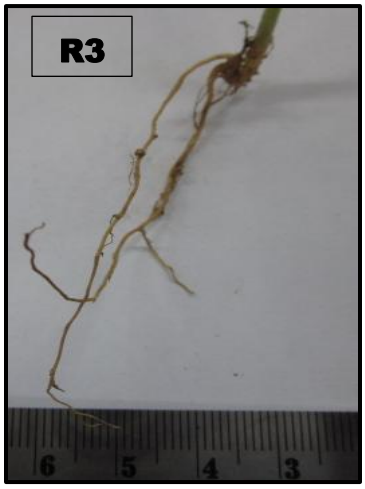


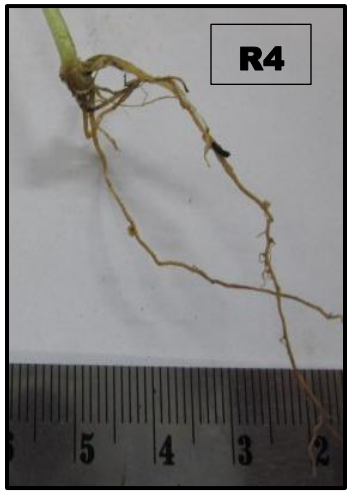


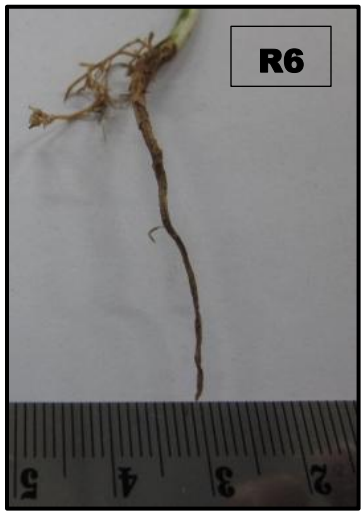
D) Mung bean [Treated NPK (NPK)]

i) Before flowering

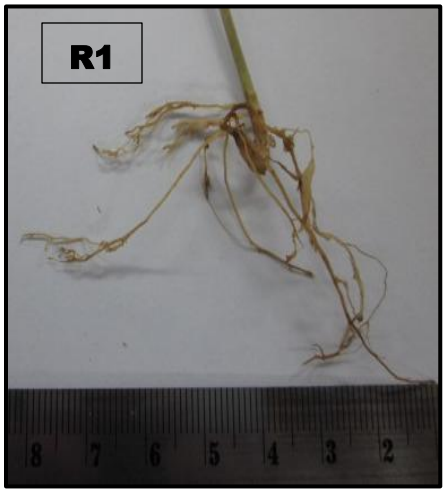


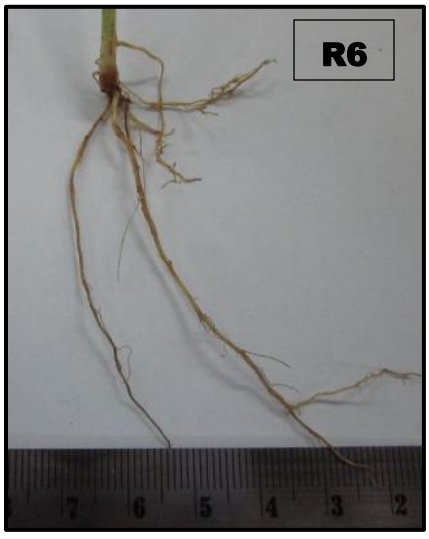




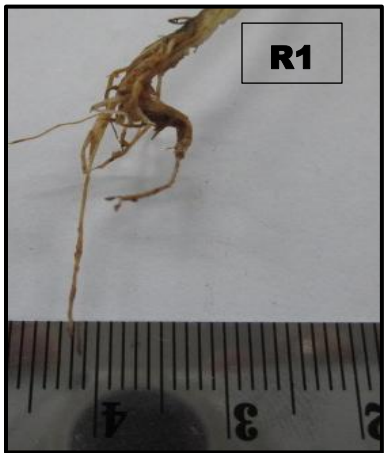


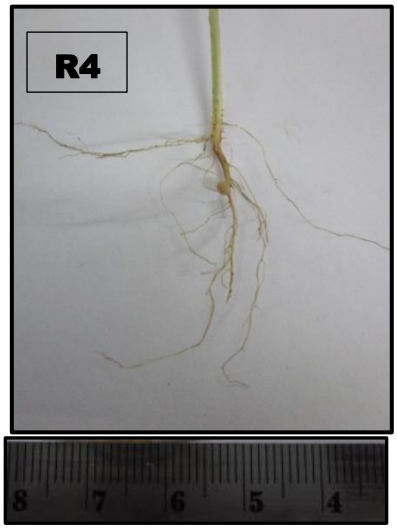
ii) Initiation of flowering

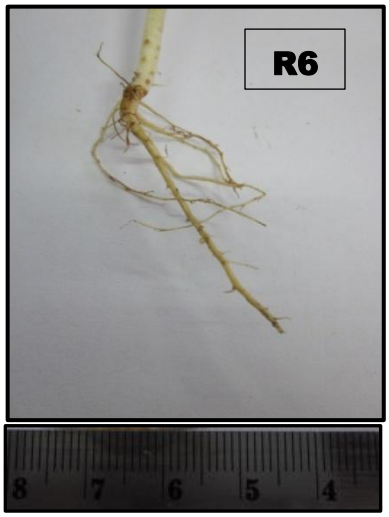




iii) Pod formation







iv) Maturity